Towards Optimisation of White-rot Fungi Bioremediation

A thesis
submitted in partial fulfillment
of the requirements for the Degree
of
PhD in Environmental Science
in the
University of Canterbury
by
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University of Canterbury
2004

Trametes versicolor

Statutory Declaration

Bioremediation research and its complexity and multidisciplinary approach requires team-work. Therefore the thesis presented has been carried out in teams of varying sizes, which has always involved me and my colleagues as also noted in the foreword of each chapter.

Throughout the thesis I have presented here for examination, I have played a leading role in the planning and the initiation of the research, the conduct of the experiments, the analysis of the results, the theory development and the writing of the papers. I have been either the principal investigator (Chapters 2,4-9) or at least an equal collaborator (Chapter 3).

I declare that I have carried out, directed and/or significantly assisted in all the work submitted here for consideration for the Degree of PhD in Environmental Science. The assistance I have received is clearly described in the foreword of each chapter and reflected in the joint authorship of the papers and via the acknowledgements in those papers.

The following researchers have been part of the teams involved in the work presented for this thesis.

Ms Kirsty Boyd Wilson, HortResearch, Lincoln

Ms Joanne Harris, formerly HortResearch, Lincoln

Ms Jenness Guthrie, HortResearch, Lincoln

Ms Sally Allan, formerly HortResearch, Lincoln

Dr Chris Frampton, formerly Lincoln University, Lincoln

Dr Laurence Boul, Wool Research Organisation of New Zealand, Lincoln

Dr Glyn Francis , Crop & Food Research, Lincoln

Dr Trevor Webb, Landcare Research, Lincoln

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Dr Grant Northcott, HortResearch, Ruakura

Mr James Pinfold, HortResearch, Ruakura

Prof Steven Aust, Utah State University, Logan, USA

Dr Richard Lamar, Earthfax Development, Logan USA

Especial mention needs to be made to the Lincoln-team and their hands-on input with Ms Kirsty Boyd-Wilson my colleague and friend of long standing.

Signed M. Walter 30.1.04

(Monika Walter, The Candidate)

Signed Hailotonu 30.01.04

(Justice of Peace)

JUSTICE OF THE PEACE FOR NEW ZEALAND J.P.

Summary

Towards optimisation of white-rot fungi bioremediation

Background

New Zealand has a large number (approx. 8000) of sites contaminated by persistent chemicals, of which approximately 10% arecontaminated with pentachlorophenol (PCP) aa a legacy of former timber treatment sites. The fungicide PCP was used extensively by the forestry industry from the late 1940s to prevent sapstaining of wood. New Zealand was a heavy user of industrial grade PCP because of the predominance of radiata pine (*Pinus radiata*) which is a soft timber and more susceptible than most tree species to sapstain fungi.

International research has shown that soils contaminated by such xenobiotics may be ameliorated using white-rot fungi. To avoid the uncertainties associated with the release of foreign organisms into the New Zealand environment, as legislated by the Hazard Substances and New Organisms Act (HSNO) and governed by the Environmental Risk Management Authority (ERMA), a national research initiative was undertaken in 1996 to study the potential of New Zealand native white-rot fungi for bioremediation.

Native white-rot isolates were (1) collected (bioprospecting), (2) selected for their ability to degrade xenobiotics – in the initial phase using PCP as the model compound and (3) studied for their mechanisms and pathways of degradation. Organic waste materials were also evaluated for their suitability to serve as a carrier for fungal augmentation to polluted soil. This PhD study formed part of this larger national research programme, with very close interaction between the different researchers and research activities. The aim of this thesis was to optimise white-rot bioremediation of New Zealand isolates. The work described here was led by me, and the principal results are mine. Selected organisms were evaluated for PCP loss and breakdown in soil. Soil

limiting factors (such as soil type, moisture, temperature, pollutant concentration) affecting colonisation of augmented isolates were identified. These laboratory results then were transferred into the field and PCP degradation studied using proto-type biopiles.

Experimentation

Screening experiments. From a pool of 367 white-rot fungi native to New Zealand (over 77 genera), isolates were screened for their PCP bioremediation potential. Fungi were tested for their ligninolytic activity (dye discoloration and wood decay), tolerance to temperature, resistance to PCP, PCP degradation *in vitro* and laccase expression. Of the isolates tested, 26% showed a discolouration in the polymeric dye assay, but all caused wood decay (5 to 169 mm) on willow cuttings. All isolates survived incubation from 0 to 30°C, however, 18% and 40% did not survive incubation at 35 and 40°C, respectively. In the PCP resistance tests, 9% were able to grow on 200 mg/L PCP amended agar, of which 20 isolates were further studied for laccase expression and PCP degradation *in vitro*. All 20 isolates reduced ($P<0.05$) PCP in the liquid fraction in the absence or presence of laccase and five of the isolates produced no detectable levels of PCP. None of the screening tests were predictive for PCP degradation *in vitro*.

Growth substrate selection and mineralisation experiments. Nine New Zealand native white rot fungi were studied for their ability to grow and survive on different substrates formulated from bark, wheat straw, sawdust, apple pomace and maize products in order to identify their PCP biodegradation potential and to select a fungal carrier for bioaugmentation of polluted soils. Isolates were also evaluated for their ability to mineralize ^{14}C-PCP in liquid culture and in soil. The American fungus *Phanerochaete chrysosporium* (ATCC 24725) outgrew the native fungi on the substrates tested, but the high colonisation did not result in PCP dechlorination as measured by chloride release and PCP mineralisation. Virtually no pentachloroanisole (PCA), a toxic metabolite of PCP, was captured in the volatile fraction of *T. versicolor* isolates, whereas 75% of the volatile fraction of *P. chrysosporium* consisted of PCA, indicating different pathways and mechanisms of degradation.

PCR detection. DNA was isolated from three *Trametes versicolor* isolates in soil using bead beat extraction combined with extract clean-up and was amplified by PCR (polymerase chain reaction) using ITS-1 and ITS-2 primers. The detection limit of

mycelium in soil was dependent on isolate, soil type and mycelium type (fresh versus freeze-dried). Nested PCR greatly enhanced *T. versicolor* detection compared to single PCR amplifications. DNA from fresh mycelium was more readily amplified (approximately 10 fold) compared to freeze-dried mycelium (based on mycelial dry weights).

Soil factors. Soil colonisation was significantly dependent on isolate and soil type, ranging from sparse to complete colonisation for the three different *T. versicolor* isolates studied, with a significant interaction between soil type and fungal isolate. Soil colonisation was also affected by augmentation with SCS. The concentration of SCS was less important than the presence of SCS (>25%). Other parameters that significantly affected fungal growth were temperature, pH, soil moisture level and pollutant concentration. Linear correlation studies between soil properties (after addition of a fungal growth substrate) and growth showed that the only significant correlation that occurred for all three isolates was between mycelial cover and percentage base saturation.

Treatability studies. The main finding in these laboratory experiments was that similar levels of aged PCP residues in soils are more toxic to white-rot fungi than an equivalent spiked PCP contamination. Also, pollutant degradation followed different patterns depending on whether the contaminant is aged or added fresh. The upper limit for PCP bioremediation by *T. versiolor* was in the order of 1300-1800 mg/kg aged PCP.

Working with aged residues, high variations between samples occurred. These were overcome by stratified sampling (combining sub-samples, homogenising and re-sampling), which also halved the analytical costs. This was required to demonstrate treatment effects, but also to provide a treatability assessment within a reasonable budget for a polluted site.

Field remediation. Engineered soil cells were designed to develop proof-of-concept biopiles for white-rot bioremediation of aged PCP contaminated soil from a former timber treatment site. Soil cells were constructed to allow for forced aeration, irrigation, leachate collection and monitoring of temperature and soil moisture content. Parameters studied were the effect of a New Zealand *Trametes versicolor* isolate on PCP degradation, the effect of fungal inoculum concentration on PCP degradation and reproducibility of the experiments. PCP degradation and fungal survival were monitored at regular intervals for 2.5 years. The experiments were set up in January 2000. There was no effect of inoculum concentration and treatment effects were reproducible. PCP

residue levels declined from 800-1000 mg/kg to 4 (0-9.4 mg/kg) in a first order kinetics. Irrigation was not required during the 2.5 years of the study, nor did leachate form. The soil cells did not exceed average daily temperatures of 35°C.

Conclusion

All isolates showed ligninolytic activity by decaying willow cuttings. The polymeric dye indicating hydrogen peroxidase activity and the laccase assay identified isolates that produced certain lignin-modifying enzymes. However, neither ligninolytic activity nor growth rate nor pollutant tolerance were indicative of biodegradation potential. This demonstrates that isolates need to be characterised and tested vigourously prior to augmentation into polluted soil sites.

The soil microcosm studies, using contaminated soil from a timber treatment site, clearly showed that the New Zealand *T. versicolor* isolates mineralized PCP. Degradation of PCP in non-sterile soil was higher in the presence of white-rot fungi than in soil without white-rot fungus. This demonstrates that viable white-rot fungus is necessary for significant PCP degradation and that *T. versicolor* isolates showed PCP remediation potential. Wheat straw and SCS could be suitable carriers for New Zealand native *T. versicolor* isolates for bioremediation of PCP polluted soil sites, however, in New Zealand, sawdust is more readily available and cheaper.

For successful bioremediation in the field good colonization of the polluted soil is desirable, so the rate-limiting factors for colonization and bioremediation were identified. This and the variable nature of contaminated soil required reliable assessments (treatability studies) to optimise the bioremediation process in the field. Treatability studies were designed to select fungal isolates, to determine optimum fungal inoculum concentration and maximum contaminant concentration, and to predict degradation over time. Isolate specific effects highlight the importance of a detailed database of growth and survival characteristics under specific conditions in order to select isolates for transfer to the field.

Proof-of-concept biopiles in the field further demonstrated a New Zealand native white-rot isolate is capable of degrading PCP from 800 mg/kg to less than 50 mg/kg in 74 weeks. Additional natural attenuation for 2 more years further reduced the PCP levels to less than 5 mg/kg. In the screening tests, all isolates survived temperatures of between 0 and 30°C. As temperatures within the soil cells stayed within this range,

biopile temperatures would not have limited growth of these isolates. However, it is important to control temperature to avoid killing the fungal mycelium by over-heating. The results clearly show that the white-rot fungus *T.versicolor* can biodegrade PCP from aged soils in a field situation.

Acknowledgements

I would like to thank HortResearch (The Horticulture and Food Research Institute of New Zealand Ltd.) and the New Zealand Foundation for Research, Science and Technology for funding.

Thanks to my supervisors and advisors for guidance received during the project, namely Dr Mark Milke (University of Canterbury), Dr John Klena (University of Canterbury), Dr Chris Frampton (Lincoln University), Dr Brent Clothier (HortResearch), Dr Lawrence Boul (WRONZ), Dr Robert Chong (Massey University), Dr Pat Holland (HortResearch) and Dr Grant Northcott (HortResearch). Thanks to Dr Rich Lamar (Earthfax Development) and Dr Steven Aust (Utah State University) for their kind advice and discussions.

I appreciate the help received from local (Selwyn District Council), regional (Environment Canterbury) and national (Ministry for the Environment) government bodies. Thanks also to participating industry partners for allowing access to their contaminated sites and providing documentation on the site history.

The project would not have succeeded without technical input and hands-on-help from my colleague and friend Kirsty Boyd-Wilson. Thanks also for technical assistance from Joanne Perry, Jenness Guthrie, Chris Ford and Sally Allen. Special thanks to my husband Manfred Bürger for lending a helpful hand and power-tool during the construction of the engineered soil cells, and having fun with the wind when putting together the kit-set aluminium shed.

I'm also very grateful to my friends, family and colleagues at Lincoln who did not ask me 'when are you finished?' Instead they offered a laugh (or a joke) during the grim times and didn't mind me being grumpy or home late. Thanks to my daughter Hannah, now 13, for cooking once a week and getting better at it.

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Chapter 1

General Introduction

Aim

New Zealand has a large number (approx. 800) of sites contaminated by persistent chemicals that affect land use and threaten local and regional ecosystems (Anon. 1995). International research has shown that chemically contaminated soils may be successfully "cleaned" using white-rot fungi (Barr and Aust 1994). This process is known as bioremediation. To avoid the uncertainties associated with the release of foreign organisms into the New Zealand environment, a national research initiative was undertaken in 1996 to study the potential of New Zealand native white-rot fungi for bioremediation. Previously, native white-rot isolates had been studied for their abilities to mineralise ^{14}C-pentachlorophenol (PCP) *in vitro*. In this thesis, organisms were evaluated for their ability to decontaminate soils by degradation of PCP in the field by

1) quantifying PCP loss and breakdown associated with each fungal isolate,
2) identifying limitations (e.g., isolate, soil type, moisture, temperature, pollutant concentration) and
3) optimising fungal activity for efficient breakdown of PCP.

To achieve these goals, process monitoring tools were required for tracking survival and activity of white-rot fungi in the biopile. In addition, microcosm studies were developed to determine the soil factors influencing white-rot remediation and treatability analyses were used to predict pollutant degradation by a selected isolate in the field.

Thesis outline

This PhD research consists of five major parts:

1) Characterise fungal isolates and predict PCP degradation ability (Chapters 2 and 3)
2) Explore the use of molecular tracking methods for monitoring the survival and growth of white-rot fungi in soil (Chapter 4)

3) Determine soil factors that influence fungal colonisation by selected white-rot isolates (Chapters 5 and 6)
4) Develop bioremediation treatability analyses for selected New Zealand white-rot isolates to select best performing isolates and enhance bioremediation activity (Chapter 7)
5) Develop field remediation protocols (Chapter 8) and predict field remediation effectiveness based on treatability analysis (Chapter 9).

The individual research chapters (Chapters 2-9) are written in the format commonly used for scientific peer-reviewed publications. Each chapter, therefore, is a standalone piece of research, with its own abstract, introduction, materials and methods, results, discussion, acknowledgement and reference sections. In addition, a General Discussion (Chapter 10) is presented to discuss the overall research approach. All references cited are also listed in the Bibliography (Chapter 11).

Part of the research presented in this thesis has been used in a patent application (New Zealand patent application number 519022). Some of the research chapters have either been published, submitted for scientific publication, in preparation for publication or a considered a trade secret. A footnote at the front page of each research-chapter describes the publication status, the Journal submitted/to be submitted and the addresses of co-authors. It also indicates if a section is to be considered a trade secret. The foreword in each research chapter further identifies the contribution of the student and other researchers. All chemical residue analysis was outsourced to TELARC accredited analytical laboratories.

Additional research that has not been included in the scientific publication was presented under the heading 'Additional information' in each chapter as appropriate. For editorial consistency, chapters comprising submitted (or to be submitted) papers for publication have been re-formatted to comply with the University of Canterbury thesis guidelines, rather than the specific journal guidelines.

It should be noted that the student has been the team leader for the HortReserch fungal bioremediation group, therefore all research planning – even beyond this PhD project – was largely under her directive.

Chapter 2

Screening of New Zealand native white-rot isolates for PCP degradation

Foreword

Chapter 2 research consists of the characterisation of fungal isolates and assessing the potential ability of white-rot isolates for PCP degradation. The work presented in this chapter was carried out by several researchers, as reflected in the number of co-authors in the footnote. Poly R-478 and wood decay studies were conducted by A. Slade and S. Sivakumaran, respectively, at Palmerston North. All other research carried out by M. Walter at Lincoln with technical assistance from K.S.H. Boyd-Wilson and J.M. Guthrie. In particular, the input from J.M. Guthrie with respect to the PCP degradation potential and laccase expression should be noted. The former (PCP degradation *in vitro*) was conducted under supervision of M. Walter. The latter (enzymology) component form part of J.M. Guthrie's PhD, supervised by E. Parker, Massey University. PCP residue analysis was conducted by D. McNaughton. Protocols regarding to PCP extraction and analysis can be found in Appendix Chemical Residue Analysis. It should be pointed out,

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Slightly modified publication by

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Screening of New Zealand native white-rot isolates for PCP degradation. Bioremediation Journal (2003) Volume 7, Issue 2, Page 119-128

that the student, M. Walter, was in charge of designing all research carried out at Lincoln, with considerable input also into the wood decay studies. In addition, M. Walter not only is the senior author of the publication but also has conducted all statistical analysis (using the raw data), with guidance from the biometrician C.M. Frampton, formerly of Lincoln University. A slightly modified version of the chapter below (excluding – of course – the section 'Additional information') has been published by Bioremediation Journal. PCP residue and laccase data are given in Appendix to Chapter 2.

Abstract

From a pool of 367 white-rot fungi native to New Zealand (over 77 genera), isolates were screened for their bioremediation potential of pentachlorophenol (PCP). Fungi were tested for their ligninolytic activity (Poly R-478, 367 isolates; wood decay, 235 isolates), tolerance to temperature (261 isolates), resistance to PCP (253 isolates), and PCP degradation potential plus laccase expression (20 isolates). Of the isolates tested, 26% showed a discolouration in the polymeric dye assay, but all caused wood decay (5 to 169 mm) on willow cuttings. In the temperature tolerance tests, all isolates survived incubation from 0 to 30°C. However, 18% and 40% did not survive incubation at 35 and 40°C, respectively. In the PCP resistance tests, 23 isolates (9%) were able to grow on 200 mg/L PCP amended agar, of which 20 isolates were further studied for laccase expression and PCP degradation *in vitro*. All 20 isolates reduced ($P<0.05$) PCP in the liquid fraction in the absence or presence of laccase and five of the isolates produced no detectable levels of PCP. None of the screening tests were predictive for PCP degradation *in vitro*. The requirements to build a database to select superior white-rot fungal isolates for bioremediation are discussed.

Keywords: bioremediation potential, *Trametes versicolor*, wood decay, polymeric dye-assay, PCP resistance, PCP degradation, laccase

Introduction

The ligninolytic enzymes of white-rot fungi have been associated with the biodegradation of xenobiotics (Pointing, 2001). Degradation of pentachlorophenol (PCP) by the American fungi *Phanerochaete chrysosporium* Burds. and *Phanerochaete*

sordida (Karst.) Erikss. & Ryv. has received particular attention (Mileski et al., 1988; Lamar et al., 1990a; 1990b; Lamar and Dietrich, 1990; 1992; Lamar et al., 1993). However, other white-rot fungi, such as *Trametes versicolor* (L.: Fr.) Pilat, have also shown potential as PCP degraders (Roy-Arcand and Archibald, 1991; Seigle-Murandi et al., 1991; 1993; Alleman et al., 1992; Lamar and Dietrich, 1992, Ricotta et al., 1996; Walter et al., 2003).

New Zealand has an estimated 800 PCP polluted soil sites deemed in need of remediation (Anon., 1995) which could benefit from the introduction of suitable degrader white-rot fungal isolates. However, the introduction of non-native fungi such as *Phanerochaete chrysosporium* into New Zealand is not permitted under New Zealand legislation (The Hazardous Substances New Organisms Act 1996, HSNO, http://www.hsno.govt.nz). We therefore undertook a study to investigate the potential of New Zealand white-rot fungi for use in bioremediation.

White-rot fungi have been screened for their bioremediation potential by measuring fungal ligninolytic activities, pollutant resistance and/or pollutant degradation (Lin et al., 1991; De Jong et al., 1992; Alleman et al., 1993; Lamar et al., 1999). The objectives of the current work were to identify the bioremediation potential of New Zealand white-rot isolates and to describe physiological, biochemical and ecological characteristics of these isolates. Therefore, a series of screening experiments measuring ligninolytic activity, PCP tolerance and degradation, as well as temperature tolerance was set up to determine the PCP soil remediation potential of New Zealand native white-rot fungi.

Materials and methods

Fungi and inoculum preparation

New Zealand white-rot isolates were obtained from bioprospecting and as gifts from Landcare Research, Auckland, and Forest Research, Rotorua. A total of 481 isolates were collected from at least 77 different genera, but not all isolates could be identified. The two American isolates *P. chrysosporium* (ATCC 24725) and/or *P. sordida* (ATCC 90628) were included in the screening experiments for control purposes. All isolates were maintained as mycelial plugs in 7 mL bijou bottles (Samco Laboratories) of sterile distilled water at room temperature. Fungal inoculum was produced by transferring a mycelial plug from the bijou bottle onto a malt extract agar

(MEA, Merck) plate. Plates were incubated in the dark at 25°C for 6 and 14 days for the fast and slow growing isolates, respectively. Culture plates were then stored at 4°C for up to 4 weeks and used for further subculturing or as a source of inoculum in the various screening experiments. Fungal subcultures were taken from MEA plates for no more than 3 generations to avoid degeneration.

For the PCP degradation experiments, isolates were grown on a fungal growth substrate (SCS) consisting of fresh *Pinus radiata* sawdust-cornmeal-starch mixture (Leštan et al., 1996). SCS was adjusted to 50% gravimetric water content, weighed (20 g) into vented glass petri dishes (85 mm diameter) and sterilised by autoclaving at 121°C and 103.5 kPa for 1 h over 2 successive days. The sterile SCS was then inoculated with an 8 mm mycelial plug and incubated for 3-4 weeks at 20 or 25°C in the dark and used for inoculum in PCP degradation experiments.

Ligninolytic activity

Ligninolytic activity was measured by polymeric dye decolourisation using Poly R-478 (anthrapyridone chromophore) according to the method of Glenn and Gold (1983). Poly R-478 was used instead of Poly R-481 because the original polymeric dye was no longer available. Isolates were assessed for presence or absence of discoloration. Screening was conducted in two experiments for a total 367 isolates.

Ligninolytic activity was also measured by assessing wood decay of willow cuttings. Fresh stems of willow *Salix schwerinii* E. Wolf cv. Kinuyanagi PN386, 20 mm in diameter and 180 mm in length, were cut from the nursery, at HortResearch, Aokautere, Palmerston North. The stems were immediately inoculated at one end with white rot fungi from agar culture and taped with masking tape. The other end of the stem was immersed (30 mm) in a container containing deionised water and incubated at room temperature. The deionised water was changed regularly to prevent mould/bacterial contaminations on the emerged stems. After 3 months incubation, stems were spilt longitudally and the amount of decay measured in mm. All 235 isolates studied were randomly selected from the culture collection and tested in triplicates.

Tolerance to temperature

In conjunction with the PCP resistance tests (below), 261 isolates were selected based on genera and origin as well as wood decay. Isolates were studied for their

temperature tolerance to determine their upper and lower temperature limits for growth. MEA plates were centrally inoculated with an 8 mm mycelial plug (mycelial side down) and incubated at 0, 5, 20, 25, 30, 35 and 40°C. There were two replicate plates for each isolate at each temperature. Mycelial growth diameter (mm) was measured in two perpendicular directions after 3 and 7 days of incubation. If little or no visible growth was observed after the 7 days incubation, isolates were further incubated at 25°C for 7 additional days. Isolates that did not resume growth were assessed as dead. Temperature in the incubators was monitored with dataloggers (Tinytalk). Screening was conducted over 15 experiments handling 50 to 900 plates per experiment. The two American isolates were not included in this test for quarantine concerns.

Resistance to PCP

Tests to determine resistance to PCP were conducted to identify New Zealand white rot isolates with a high tolerance to the pollutant. The tests were carried out on agar plates amended with PCP (Aldrich). PCP was dissolved in acetone and diluted with autoclaved MEA at 50-55°C to give a range of PCP concentrations (0, 10, 20, 30, 40 and 50 mg/L PCP). Agar was dispensed (20 ml) into vented sterile plastic petri dishes (85 mm diameter). The volume of acetone added (5 ml/L agar) was constant for all concentrations. The nil-control consisted of straight MEA plates (nil PCP and nil acetone). Acetone-control refers to the 0 mg/L PCP plus acetone carrier solvent. Plates were allowed to dry (lids closed) for 1-2 days at room temperature in the fumehood prior to inoculation with two 6 mm plugs of inoculum (mycelial side down) positioned on opposite margins of the plate. Mycelial growth was measured from the inner edge of the plug to the centre of the plate after 3, 7 and 14 days incubation at 25°C in the dark. Growth on the PCP amended agar was expressed as a percentage of the growth on the nil-control plate. There were two replicate plates for each isolate at each concentration.

From the 261 native isolates used in the temperature screening test, 8 isolates were discarded due to contamination. One-hundred-sixty-three native isolates were tested for PCP tolerance against the complete range of PCP concentrations (including acetone-control). An additional 90 isolates (including *P. sordida* and *P. chrysosporium*) were screened for PCP tolerance at the 50 mg/L PCP concentration in comparison to the nil-control. All isolates showing growth at the 50 mg/L PCP level were also tested for PCP tolerance at 100, 150 and/or 200 mg/L. At these higher PCP concentrations only

presence or absence of mycelial extension was recorded. Screening was conducted over 18 experiments, handling 50 to 300 plates per experiment.

PCP degradation in vitro

Twenty randomly selected isolates (Table 2.1) tolerant to 200 mg/L PCP in agar were tested for their ability to degrade PCP in liquid culture. Five grams (fresh weight) of colonised SCS inoculum was introduced into 100 mL of nitrogen limited medium (Dodson et al., 1986) containing 50 mg/L PCP (dissolved in acetone) in a 250 mL Erlenmeyer flask. Flasks were incubated statically at room temperature (20-25°C) in the dark. During incubation laccase activity was monitored at regular intervals as described below. After 42 days the mycelium was filtered under vacuum through filter paper (Whatman No. 1) and the filtrate analysed for PCP by HPLC. For each isolate two replicate flasks, an un-inoculated PCP control and a fungus (isolate HR131) control were analysed for residual PCP. The latter consisted of liquid medium and fungal inoculum without PCP but acetone added.

PCP extraction and analysis of liquid cultures. All solvents and reagent chemicals were high purity (Mallinckrodt Nanograde, Merck-BDH analytical grade). Ultrapure water was prepared by distillation followed by a Milli-Q-system (Millipore, Milford, MA, USA). A certified standard of PCP (99% purity, Dr Ehrenstorfer GmbH, Augsburg, Germany) was used to prepare calibration standards for HPLC analysis.

Measured aliquots of filtered liquid culture samples (50-90 ml) were extracted with 100 mL acetone/hexane (2:3 v/v) by shaking for 60 minutes on a flat bed shaker (300 cycles/minute). After shaking, 150 mL of distilled water was added to separate the hexane phase containing extracted PCP and 0.25 mL was transferred to a 2 mL glass HPLC vial. Twenty microlitres of iso-butanol was added as keeper solvent and hexane was removed under a gentle stream of nitrogen gas at room temperature. Then 1.6 mL methanol/water (80:20 v/v) was added to each vial and the samples were stored under refrigeration until analysis by HPLC. PCP recovery during sample workup was calculated from control samples spiked with a known amount of PCP and was typically 90 to 100%. The amount of PCP measured in a corresponding batch of samples was corrected by the appropriate recovery factor.

Liquid chromatographic separation and detection of PCP were performed with a Shimadzu LC-10A liquid chromatography system and Shimadzu SPD-10AV UV-vis detector at 280 nm. Chromatography was performed at 35°C with an isocratic methanol/water/acetic acid mobile phase (81/18/1% by volume) and 5 µm, 150 x 4.5-mm-i.d.Luna C_8 reversed phase column (Phenomenex NZ Ltd). The column flow rate was 1.0 mL/min and injection volume 10 µL. Six calibration standards (50, 20, 10, 5, 2.5, 1 µg/mL) were analysed with each batch of samples and the samples quantified against the constructed calibration curve.

Laccase activity. Laccase activity was monitored regularly (up to 23 measurements/isolate/rep) during the 42 day incubation period. Small amounts of liquid (300 µL/measurement) were removed under aseptic conditions and laccase was assayed as peroxide-independent degradation of 2,6-dimethoxyphenol at pH 4.5 at 450 nm (De Jong et al., 1994).

Statistical analysis

Isolate differences were demonstrated using descriptive statistics, except the effect of isolate on PCP degradation *in vitro* was assessed using Analysis of Variance (ANOVA, Systat 6.1). Isolate differences were described using Fisher's Least Significant Difference test (LSD). For the correlation analyses of the different screening tests (using the means) Pearson's Correlation tests (Minitab Version 12.1) were used.

Results

Ligninolytic activity

For the 367 isolates tested, using the polymeric dye assay Poly R-478, 95 isolates (26%), including *P. chrysosporium*, showed obvious discolouration after 7 – 11 days of incubation. For the 235 isolates tested in the lignin degradation assay using willow cuttings, all isolates caused wood decay ranging from 5 to 169 mm. Sixty-seven isolates (29%) caused wood decay greater than 84.5 mm ($^1/_2$ of the maximum wood decay), with 34 isolates (14%) rotting at least 126.75 mm ($^3/_4$ of the maximum wood decay) of the wood in the willow cuttings. Wood decay is summarised in Table 2.2 for the 23 isolates with the highest PCP tolerance.

Resistance to PCP

Of the 253 white-rot isolates tested, 95 New Zealand native isolates (38%) produced viable growth on 50 mg/L PCP. The two American isolates, *P. chrysosporium* and *P. sordida*, did not grow on 50 mg/L PCP amended agar. Total mycelial extension ranged from as little as 2 mm to 40 mm during the two week incubation period, with the 40 mm extension being the maximum growth measurable under the experimental conditions. From the 95 New Zealand isolates tolerant to 50 mg/L PCP, 23 isolates (9% of the 253 isolates) maintained viable growth on 200 mg/kg PCP amended agar (Table 2.1).

Tolerance to temperature

Growth rate on agar was highly variable between isolates. Mycelial growth after 7 days incubation at 30°C varied between 1 mm and 77 mm (complete coverage of the plate). Whilst variation in growth is expected between genera and species, substantial variations in growth also occurred within species. For example, growth for *Trametes versicolor* isolates varied from 7 mm to 77 mm during the 7 days incubation at 30°C. Similarly, optimum temperature for growth as well as tolerance to temperature extremes was isolate specific, and variable within and between species. Of the 261 isolates tested for temperature tolerance, 78 (30%) did not grow at 0°C, but all resumed growth following incubation at 25°C, and 74 (28%) isolates did not grow at 5°C, but again all resumed growth at 25°C incubation. All isolates were able to grow at temperatures of 20, 25 or 30°C. At 35°C, 83 isolates (32%) did not grow, but 36 isolates resumed growth during subsequent incubation at room temperature. At 40°C, 201 isolates (77%) did not grow, but 96 isolates resumed growth during subsequent incubation at 25°C. Two isolates (unidentified isolates HR140 and HR169) showed similar growth at 30, 35 and 40°C. Results for temperature tolerance and optima are presented in Table 2.2 for the 23 isolates with the highest PCP tolerance as indicated in Table 2.1.

Table 2.1. White-rot isolates resistant to PCP on 200 mg/L PCP amended agar

Fungus (isolate code[a])	Species	Source (other code[b])	Actual growth (mm)[c]	Growth (%) compared to nil-control[d]
HR145*	*Abortiporus biemmis*	HortResearch, NZ	4.1	20.9
HR345*	*Oudemansiella australis*	Forest Research, NZ (FRI 238)	8.7	51.5
HR226*	*Peniophora sacrata*	Forest Research, NZ (FRI 36B)	10.2	27.7
HR235*	*Peniophora sacrata*	Forest Research, NZ (FRI 36K)	2.0	5.4
HR240*	*Peniophora sacrata*	Forest Research, NZ (FRI 36P)	9.7	26.4
HR241	*Peniophora sacrata*	Forest Research, NZ (FRI 36Q)	8.7	48.6
HR316	*Rigidoporus catervatus*	Forest Research, NZ (FRI 202)	20.7	56.1
HR348*	*Stereum fasciatum*	Forest Research, NZ (FRI 197)	9.7	56.5
HR192*	*Trametes* sp.	HortResearch, NZ	11.0	60.0
HR196*	*Trametes* sp.	HortResearch, NZ	9.7	51.0
HR197*	*Trametes* sp.	HortResearch, NZ	10.7	63.2
HR131*	*Trametes versicolor*	HortResearch, NZ (Culture A[e])	16.7	41.8
HR154*	*Trametes versicolor*	HortResearch, NZ (Culture B[e])	14.3	8.8
HR160*	*Trametes versicolor*	HortResearch, NZ (Culture C[e])	18.6	50.3
HR275*	*Trametes versicolor*	Forest Research, NZ (FRI 75A)	29.0	78.4
HR277*	*Trametes versicolor*	Forest Research, NZ (FRI 75C)	7.5	46.9
HR445*	*Trametes versicolor*	Landcare Research, NZ (PB86/097a)	8.2	48.5
HR112*	Unknown	HortResearch, NZ	14.5	39.2
HR122*	Unknown	HortResearch, NZ	9.2	25.0
HR152*	Unknown	HortResearch, NZ	1.7	4.7
HR577*	Unknown	HortResearch, NZ	9.2	25.0
HR588	Unknown	HortResearch, NZ	8.0	37.5
HR589*	Unknown	HortResearch, NZ	5.0	12.8

[a] HortResearch Culture Collection Code
[b] Corresponding Culture Collection Code from supplier
[c] Actual growth (mm) on 50 mg/L PCP amended agar determined by mycelial extension after 14 days incubation at 25°C in the dark
[d] Percentage growth compared to nil-control on 50 mg/L PCP amended agar after 14 days incubation at 25°C in the dark
[e] Deposited at Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia with Accession numbers NM02/27875, NM02/27876, and NM02/27877 for Culture A, Culture B, and Culture C, respectively.
* Randomly selected for further PCP degradation studies *in vitro*. A maximum of 20 isolates could be selected due to resource, equipment and space limitations.

Table 2.2. Temperature tolerance at 35 and 40°C and lignolytic activity as measured by wood decay, Poly R-478 and laccase expression in the presence of PCP for the 23 selected PCP tolerant isolates

Fungus (code[a])	Temperature tolerance			Ligninolytic activity		
	Maximum growth (°C)	Growth (G) at 35°C	Growth (G) at 40°C	Poly R-478 colour reaction	Wood decay (mm)	Laccase detected[b]
HR112	25	G	G	No	111.0	Yes
HR122	25	G	G	Yes	163.3	Yes
HR131	30	G	G	Yes	136.7	Yes
HR145	25	G	-	No	nt	No
HR152	25	G	G	Yes	102.7	No
HR154	30	G	G	Yes	138.4	Yes
HR160	25 - 30	G	G	Yes	142.7	Yes
HR192	25	-	-	Yes	19.0	No
HR196	25	-	-	Yes	100.0	No
HR197	25	-	-	No	14.7	No
HR226	25	-	-	No	127.0	Yes
HR235	20	-	-	No	66.7	Yes
HR240	20	-	-	No	106.7	Yes
HR241	25	-	-	No	84.7	nt
HR275	30	G	G	No	51.7	Yes
HR277	25	-	-	Yes	7.0	No
HR316	25	G	G	No	156.0	nt
HR345	25	-	-	No	nt	No
HR348	25	G	-	No	138.7	No
HR445	25	-	-	Yes	93.3	No
HR577	30	G	G	nt	73.0	Yes
HR588	25	G	-	nt	nt	nt
HR589	35	G	G	nt	nt	No

[a] HortResearch Culture Collection Code

[b] Laccase detected during 42 days of incubation in the *in vitro* PCP degradation test

nt = not tested

PCP degradation in vitro

Over the 42 day incubation period, all 20 isolates tested significantly ($P<0.05$) reduced PCP in the liquid fraction when compared to the PCP-control (Figure 2.1). For five of the isolates PCP could not be detected in the liquid fraction. PCP degradation occurred irrespective of mycelial growth in the liquid fraction. The fungal control (HR131) and isolates HR145, 152, 192, 197, 226, 235, 240, 277, 445, and 589 all formed a mycelial mat during 14 days of incubation, whereas isolates HR112, 122, 131, 154, 160, 275, and 577 produced a mycelial mat after 14 days of incubation and isolates HR196, 345, and 348 did not form a mycelial mat.

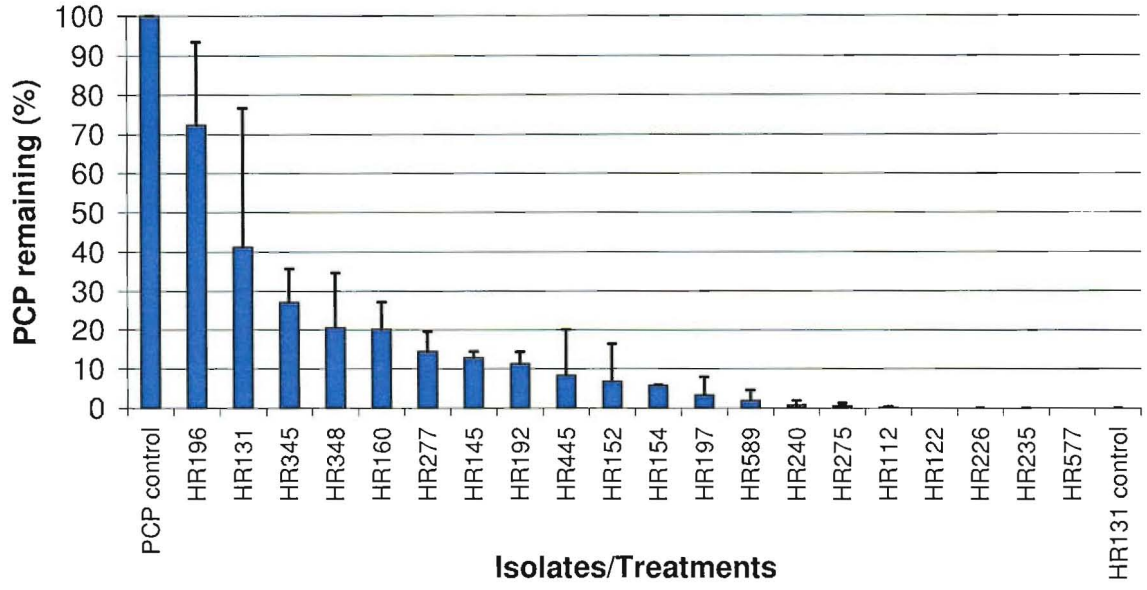

Figure 2.1. PCP remaining in the liquid fraction after 42 days stationary incubation with white-rot fungi at room temperature. Error bars show the standard deviation of the means.

Laccase activity within genera tended to follow a similar pattern, as shown for representative isolates of *P. sacrata* and *T. versicolor* in comparison with 3 unidentified isolates (Figure 2.2). Ten of the white-rot isolates produced laccase at some stage over the 42 days of the experiment (Table 2.2). Mycelial growth observations showed that laccase activity after 14 days (for all isolates that produced this enzyme) appeared to correspond to formation of the mycelial mat on the surface of the liquid. If isolates did not form a mycelial mat, measured laccase activity decreased after 14 days.

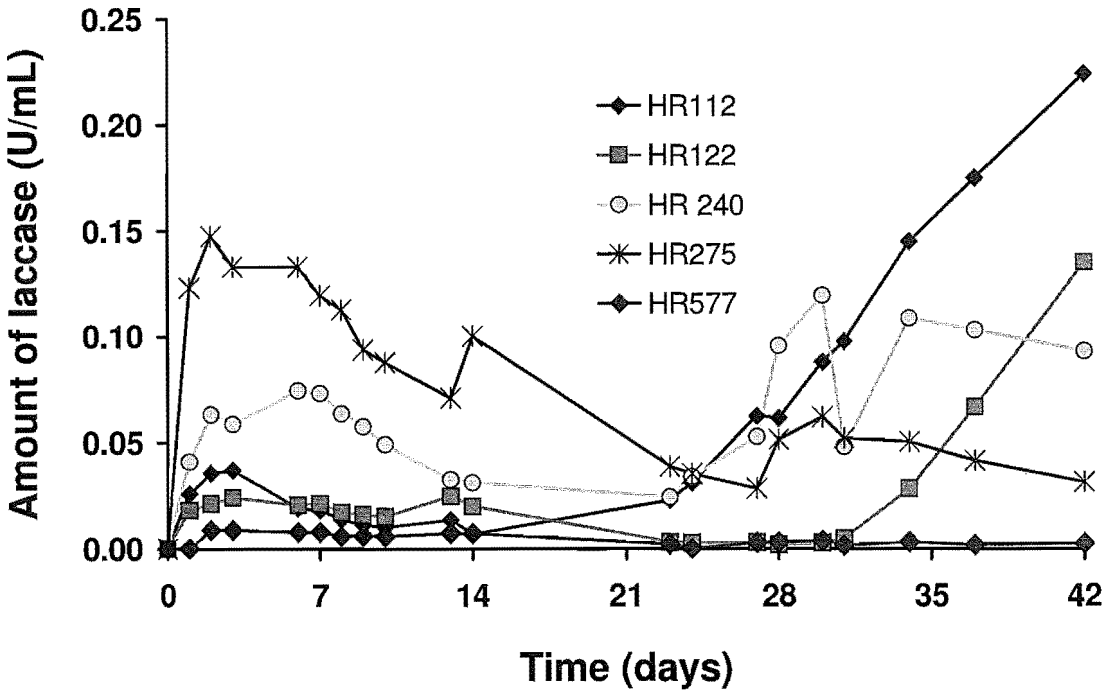

Figure 2.2. Laccase activity over 42 days incubation in 50 mg/L PCP nutrient deficient medium for selected New Zealand white-rot isolates. Pooled standard error of the means SE = 0.01 U/mL. (HR240 = *P. sacrata*; HR275 = *T. versicolor*; HR112, HR122, HR577 = unidentified isolates).

Correlation analyses

Results of correlation analyses between the different screening tests are shown in Table 2.3. None of the screening tests were predictive/indicative for PCP degradation *in vitro*.

Table 2.3. Correlation coefficients between screening tests

	PCP degradation *in vitro*	**Poly R-478**	**Wood decay**	**Temp. optimum for growth**	**Temp. tolerance (40°C)**	**PCP tolerance on agar (50 mg/L)**
Poly R-478	0.328	-	-	-	-	-
Wood decay	0.129	0.203	-	-	-	-
Temperature optimum for growth	0.000	0.663*	0.231	-	-	-
Temperature tolerance (40°C)	-0.082	0.428	0.478	0.389	-	-
PCP tolerance on agar (50 mg/L)	0.311	0.132	-0.410	0.046	-0.115	-
Growth on MEA (nil-control, 30°C)	0.107	0.604*	.049	0.609*	0.670*	0.045

* significant P<0.05

Discussion

Bioremediation potential of white-rot fungi is well documented and summarised in the mini-review by Pointing (2001). New Zealand native white-rot fungi were evaluated here for their bioremediation potential of PCP because of biosecurity concerns regarding the release of foreign organisms, particularly organisms not recorded in New Zealand, into the environment.

Screening experiments were conducted not only to identify the bioremediation potential of New Zealand white-rot isolates but also to describe physiological, biochemical and ecological characteristics of the isolates. This information is required to build a database for selection of superior isolates for remediation of a particular pollutant problem for a selected treatment system (Lamar et al., 1999).

The screening tests presented, were valuable for characterisation of New Zealand native isolates and also enabled identification of native isolates with PCP bioremediation potential. In particular, the high (200 mg/L) PCP resistance test allowed for effective isolate selection. Whilst resistance does not equal PCP degradation

potential, a highly resistant isolate with good PCP degradation (as further evaluated in the PCP degradation test) is a desirable trait for remediation at the higher pollutant concentrations. For example, in New Zealand, soil contamination with a concentration over 5000 mg/kg PCP is estimated to exist for more than 1000 tons of soil, excluding sludge, sawdust and general waste (Finnbogason and St. Quintin, 1994).

Ligninolytic activity as measured by presence or absence of discoloration (Poly R dye assay) was employed as a preliminary screen. The Poly R dye decolourisation rate was found to be a good indicator for peroxidative activity, however the assay is not sensitive to laccase (De Jong et al., 1992). Therefore wood decay was also assessed as a measure of lignolytic activity (Setliff and Eudy, 1980). Both assays allowed a clear differentiation of the isolates tested, but wood decay was not dependent upon presence or absence of peroxidase activity. No histological examinations were undertaken to determine the type of decay pattern. Two distinct decay patterns are known: (1) simultaneous removal of cell wall components and (2) selective removal of lignin and hemicellulose depending on the enzyme activites (Blanchette, 1984). Given the number of isolates studied represented over 77 genera, elucidating the enzymatic processes of the different isolates was beyond the scope of this work. Laccase was the only enzyme monitored in the *in vitro* PCP degradation experiment because of the prevalence of *Trametes* isolates, which are well known for their laccase production and have been associated with PCP degradation (Tuomela et al., 1999). Laccase activity within genera seemed to follow a similar pattern according to the 5 *T. versicolor* and 3 *P. sacrata* isolates studied. All other isolates included in the *in vitro* PCP degradation screening test either remained unidentified or singly represented a species. It was beyond the scope of this initial screening experiment to determine laccase expression between and within species. The purpose of the experiment was to identify the biodegradation potential of these PCP tolerant isolates.

PCP resistance tests were carried out to determine the tolerance of New Zealand white-rot isolates. Measuring hyphal extension rate on PCP amended MEA, Lamar et al. (1990b) reported a daily growth rate of 34.9, 5.7 and 4.6 mm/day for the most tolerant isolate (*P. sordida* strain 8) at 0, 20 and 25 mg/L PCP, respectively. This compares to a daily growth rate of the second most tolerant *T. versicolor* (isolate HR160) of 6.35, 2.88 and 1.33 mm/day at 0, 20 and 50 mg/L PCP. (The most tolerant isolate HR275 was screened at 0 and >50 mg/L PCP concentrations). In our study, the American fungi did not grow at the 50 mg/L PCP level, but 38% of the tested native NZ isolates grew at 50

mg/L PCP and 9% at 200 mg/L PCP, indicating the New Zealand isolates have a high level of tolerance to PCP.

In PCP resistance testing, the actual PCP dose per milligram mycelium has been reported to be more important than the PCP concentration (mg/L) (Alleman et al., 1992). However, the screening method proposed by Alleman et al. (1993) did not result in clear inhibition zones (in a preliminary method evaluation) studying native fungi, but did for the American fungi (data not presented). Therefore the agar method was used with a small inoculum plug (6 mm) as increasing mycelial biomass causes an increase in pollutant tolerance (Alleman et al., 1992). This is supported by our observations. At the >50 mg/L PCP concentrations, the fungal colonies were slow to establish, but growth rate gradually increased with increasing colony size. If the inoculum plug was inoculated mycelial side upwards (rather than facing the agar surface) colony formation occurred more readily on the PCP agar (data not presented).

Lamar et al. (1999) reported that white-rot hyphal extension rate and sensitivity to the contaminant showed potential for predicting bioremediation performance for degradation of PCP. This, however, could not be confirmed in this study as no correlation was found between PCP degradation *in vitro* and growth rate or PCP tolerance of the tested isolates. These differences could be due to the range and number of organisms studied: The New Zealand study only investigated Basidiomycetes, whereas Lamar et al. (1999) included Basidiomycetes and Hyphomycetes. Lamar et al. (1999) studied 13 strains representing 13 Basidiomycetes species in comparison to our 20 isolates from at least 6 different species. Further research will be required to evaluate the conclusions by Lamar et al. (1999).

In this study, PCP degradation *in vitro* was assessed by compound disappearance from the liquid fraction. The main route of toxin removal remains unclear. However, in related experiments using ^{14}C-PCP (Walter et al., 2003), the distribution of ^{14}C (expressed as percentage of radioactivity initially added from various fractions) for native white-rot fungal isolates (including HR131, HR154, HR275, HR445) in a liquid culture experiment ranged from 10.9-29.8% for the culture filtrate, 3.4-6.6% for the extract of the solid fraction, 3.0-7.9% for the combusted extracted solid fraction, 2.0-16.8 for the CO_2 fraction, and 26.2-78.9% for the volatile trap. Total mass recovery ranged from 83.5 to 103.4%. The research by Walter et al. (2003) clearly demonstrated that PCP was mineralised by New Zealand white-rot fungi to CO_2 and/or degraded to water soluble compounds.

Neither the presence or absence of measured laccase, nor the total amount of laccase produced (data not presented), correlated with PCP decline in the liquid fraction, implying that other enzymatic processes may have been involved in the degradation process (Nerud et al., 1991; McAllister et al., 1996; Ryu et al., 2000). Ryu et al. (2000) concluded that lignin peroxidase, manganese peroxidase and laccase are not essential in the biodegradation of PCP by white-rot fungi. However, a wide variety of xenobiotics (including phenols, anilines and their substituted products) have been transformed through laccase activity (Gainfreda et al., 1999). Laccases are polyphenol oxidases (ie able to oxidize a phenolic substance) and the main role of fungal laccases is to depolymerise lignin (Gainfreda et al., 1999). For New Zealand white-rot fungi, further research on production of enzymes by the isolates studied and the role of laccase and the various laccase isozymes in PCP degradation needs to be carried out. The mycelial growth observations showed that laccase activity after 14 days (for all isolates that produced this enzyme) appeared to correspond to formation of the mycelial mat on the surface of the liquid medium, implies that the laccase measured was expressed constituitively during growth.

In conclusion, the objectives of this work, the identification of the bioremediation potential of New Zealand white-rot isolates and their description of physiological, biochemical and ecological characteristics, were met using a series of screening experiments measuring ligninolytic activity, temperature tolerance, PCP resistance and degradation. Based on the PCP degradation potential and isolate characteristics, ten isolates were selected for further experiments on PCP mineralisation studies as well as finding a suitable carrier for soil inoculation (Walter et al., 2003 – Chapter 3). Research has also been conducted for three selected isolates determining soil parameters that affect colonisation of typical New Zealand soils by white-rot fungi (Boyd-Wilson et al., 2005 – Chapter 6). Current research focuses on formulation development for the fungal inoculum to polluted soil sites, optimising field remediation and identifying pathways and mechanisms of PCP degradation.

Acknowledgements

The authors would like to thank the New Zealand Foundation for Research Science and Technology for funding. We are grateful to the Forest Research, Rotorua, and Landcare Research, Auckland, for allowing us to use their white-rot fungal isolates in the

screening experiments. Thanks to Drs Adrian Spiers and Nick Waipara for identification of HortResearch isolates. We would also like to thank Drs John Christeller and Grant Northcott for scientific input relating to the enzymology and chemical residue analysis, respectively.

Additional information[A]

Pentachlorophenol (PCP, Figure 2.3) was used in New Zealand as an effective biocide to preserve timber and prevent fungal rots. PCP was used until 1988 at 70% of all forestry sawmilling sites.

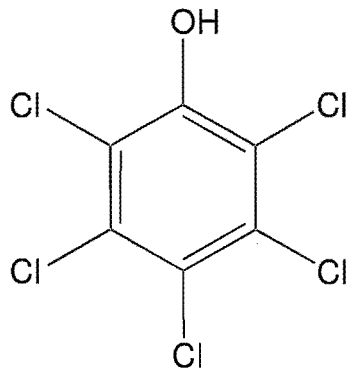

Figure 2.3. Pentachlorophenol (PCP).

The use of PCP was discontinued because of its toxicity and persistence in the environment. Approximately 800 former and/or existing sawmilling sites are deemed in need of remediation (Figure 2.4) Upon entering the environment, PCP will undergo various reactions. The fate of PCP in soil is illustrated in Figure 2.5.

[A] To the reader of this thesis, this section provides additional information that was not included in the scientific publication, but of course is part of the PhD research itself and may aid in the understanding of the results.

Figure 2.4. Sites in New Zealand contaminated with PCP and deemed for remediation (Jackman et al., 1993).

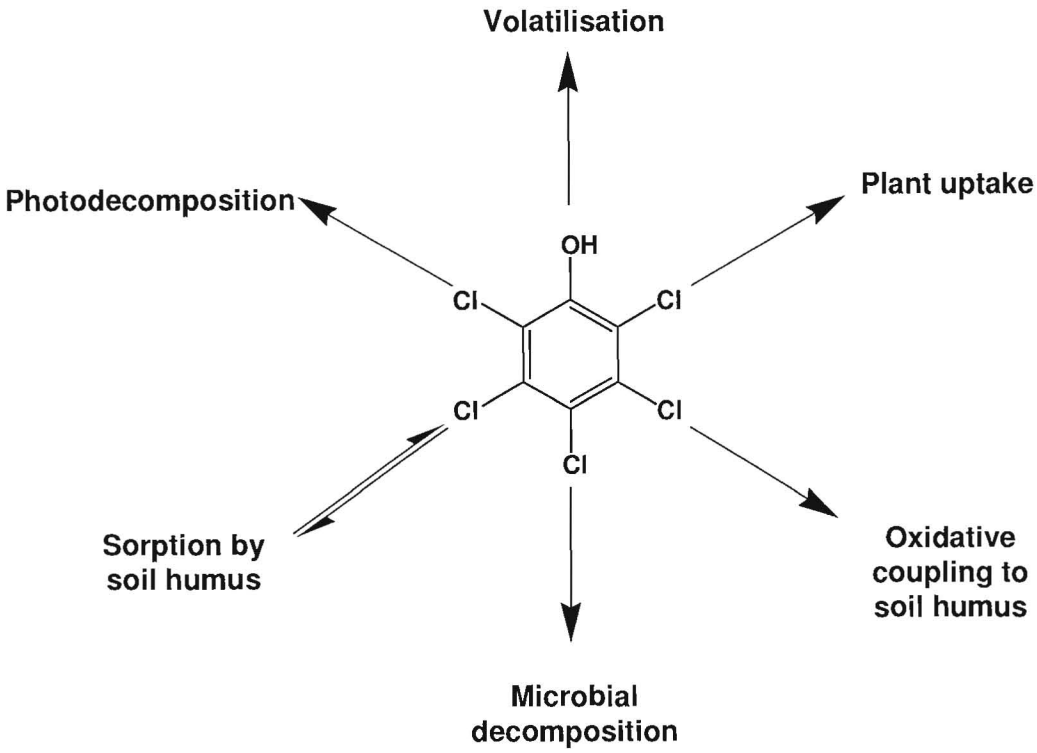

Figure 2.5. Fate of PCP in the environment.

Figure 2.6 depicts a 'snapshot' of the culture collection during the annual subbing onto 10 mL MEA slopes (universals) and storage in sterile distilled water (5 mycelial plugs/bijou bottle). Figure 2.7 shows 3 different *T. versicolor* isolates growing on PCP amended agar.

Figure 2.6. Snapshot of the extensive white rot culture collection held by HortResearch.

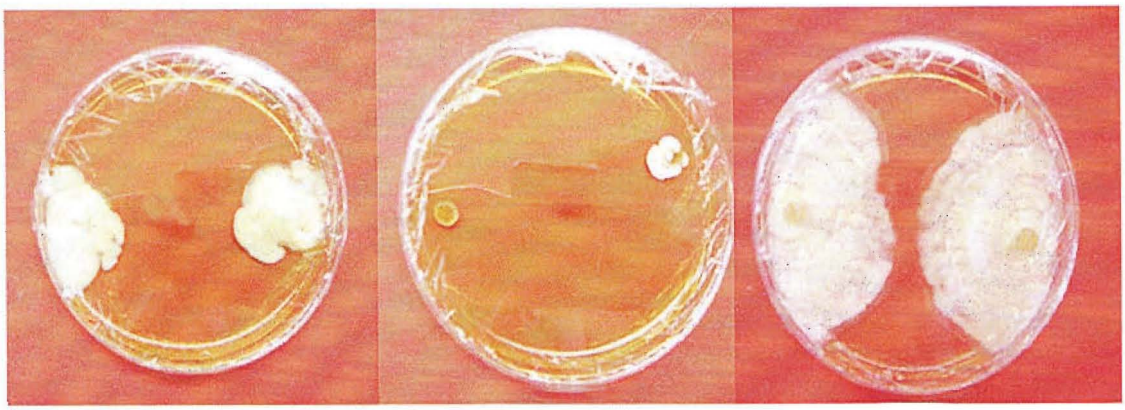

Figure 2.7. Three different *T. versiolor* isolates growing on PCP (200 mg/kg) amended agar after incubation at 30°C for 7 days. Note: Less than 1% of all fungal isolates tested grew on 200 mg/kg PCP amended agar.

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Chapter 3

Parallel development at HortResearch: Growth substrate selection and biodegradation of PCP by New Zealand white-rot fungi

Foreword

Chapter 3 is built on Chapter 2 results and further characterises New Zealand native white-rot fungal isolates with respect to their PCP degradation ability and selecting a suitable growth substrate that facilitates fungal survival and PCP degradation. The research in this chapter was a HortResearch project, led by M. Walter, conducted in parallel with the thesis. The research, although not part of the original PhD-proposal, provides complementarity to the main thrust of the PhD programme. It is included in the thesis because Chapters 2 and 3 form the backbone of the thesis. All subsequent New Zealand fungal bioremediation work is based on the results and approaches presented here. The research on PCP mineralization was conducted by Dr L. Boul, WRONZ. Detailed analytical procedures for ^{14}C-PCP residue analyses are presented in Appendix Chemical Residue Analysis. The work on growth substrate selection was carried out by Dr R. Chong and C. Ford, Massey University. PCP residue data of soil experiment 1 is presented in Appendix to Chapter 3 as an example for measurements taken during the

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Growth substrate selection and biodegradation of PCP by New Zealand white-rot fungi. Journal of Environmental Management (2004) Volume 71, Page 361-369

trials. M. Walter has led and summarised the work and written the publication.. A modified version of the chapter has been published by Journal of Environmental Management.

Abstract

Nine New Zealand native white rot fungi were studied for their ability to grow and survive on different substrates formulated from bark, wheat straw, sawdust, apple pomace and maize products in order to identify their pentachlorophenol (PCP) biodegradation potential and to select a fungal carrier for bioaugmentation of polluted soils. Isolates were also evaluated for their ability to mineralize ^{14}C-PCP in liquid culture and in soil. The American fungus *Phanerochaete chrysosporium* outgrew the native fungi on the substrates tested, but the high colonisation did not result in PCP dechlorination as measured by chloride release. Whilst *Trametes versicolor* inocula produced on wheat straw and SCS (sawdust-corn meal-starch-mix) gave highest chloride release, colonization of these two substrates as measured by biological potential was lower than the pomace and pomace-sawdust-mix. Neither lignin peroxidase nor manganese peroxidase were detected in New Zealand white-rot fungi during the experiments. Laccase was the only enzyme detected. In liquid culture, mineralisation rate was higher for *T. versicolor* isolates compared to *P. chyrysoporium.* Very little to no pentachloroanisole (PCA) was captured in the volatile fraction of *T. versicolor* isolates, whereas 75% of the volatile fraction of *P. chrysosporium* consisted of PCA. The soil microcosms studies, using contaminated soil from a timber treatment site, clearly showed that the New Zealand *T. versicolor* isolates mineralized PCP. Degradation of PCP in non-sterile soil was higher in the presence of white-rot fungi than in soil without white-rot fungus. This demonstrates that viable white-rot fungus is necessary for significant PCP degradation and that *T. versicolor* isolates showed PCP remediation potential. Wheat straw and SCS could be suitable carriers for New Zealand native *T. versicolor* isolates for bioremediation of PCP polluted soil sites.

Keywords: *Trametes versicolor, in vitro* studies, aged soil residues, mineralization, augmentation, fungal inoculum, fungal carrier.

Introduction

White-rot fungi, which grow naturally on wood, have demonstrated the ability to bioremediate xenobiotics (Barr and Aust, 1994). White-rot fungi have evolved to decompose lignin, nature's most recalcitrant substance. Lignin, a large complex insoluble molecule, is a non-repeating heteropolymer which gives structural strength to wood. White-rot fungi use a variety of enzymatic processes and biochemical reactions for the depolymerization and degradation of lignin. These extracellular oxidative processes are non-specific, thus the same mechanisms employed in lignin breakdown can be applied to environmental pollutants (Barr and Aust, 1994). The extracellular degrading system equips white-rot fungi with other advantages for bioremediation systems. They can, for example, degrade other recalcitrant anthropogenic chemicals via extracellular decomposition (Pointing, 2001). White-rot fungi are able to tolerate considerably higher concentrations of a toxic pollutant than organisms which need to uptake the toxic substance in order to decompose it (Pointing, 2001). White-rot fungi do not require preconditioning to a particular pollutant because the degrading system is usually triggered by nutrient limitation (Pointing, 2001). Leatham and Kirk (1983) showed that the regulatory effect of nitrogen on ligninolytic enzyme expression is a reflection of the inductive effect of low nitrogen levels found in wood (C:N = 200:1 to 1000:1).

In New Zealand, there are approximately 800 identified sites contaminated with pentachlorophenols (PCPs) as the result of former industrial practices (Anonymous, 1995). The aim of the research was to select native fungi capable of degrading PCP in soil and identify a suitable carrier for augmentation of white-rot fungi into polluted soil environments. Nine New Zealand native white-rot isolates were studied for their ability to colonize different substrates formulated from bark, wheat straw, sawdust, apple pomace and maize products. In addition, their ability to induce PCP degradation and to mineralize PCP in liquid culture and in soil were examined. Isolate selection was based on previous research (Walter et al., 2003 – Chapter 2).

Experimental section

Fungi

White-rot fungi isolates (Table 3.1) were maintained by growing on potato dextrose agar (PDA, Gibco) at 30°C for 6 days. Cultures were stored on slopes at 4°C at the HortResearch culture collection Palmerston North.

Selection of a fungal carrier suitable for white-rot augmentation in bioremediation

Screening experiments were set up in order to select a growth substrate for development of a suitable carrier for white-rot augmentation of polluted soil. All screening experiments were repeated at least once to test for reproducibility. The first experimental series assessed various waste materials for supporting fungal growth measured visually, and inducing PCP dechlorination - an important step in PCP degradation - measured by chloride release. Fungal isolates F1, F3, F5, F6 and F9 were studied and compared to the American white-rot fungus *Phanerochaete chrysosporium* (F2). Based on the results of the first experimental screening series, two growth substrates and one fungal isolate (F5) were selected for further studies. This second experimental series explored using duplicate tests the relationship between colonisation of the growth substrate, PCP degradation and enzyme. Experimental details are described in more detail below.

Table 3.1. White-rot fungal isolates used in this study

Fungus #	Species	Code[a]	Source (other code[b])
F1	*Junghuhnia vincta*	HR292	Forest Research, NZ (FRI 163C)
F2	*Phanerochaete chrysosporium*	HR170	Utah State University, USA (ATCC 24725)[c]
F3	*Phanerochaete cordylines*	HR469	Landcare Research, NZ (PB 88/024)
F4	*Trametes versicolor*	HR131	HortResearch, NZ (Culture A[d])
F5	*Trametes versicolor*	HR154	HortResearch, NZ (Culture B[d])
F6	*Trametes versicolor*	HR160	HortResearch, NZ (Culture C[d])
F7	*Trametes versicolor*	HR275	Forest Research, NZ (FRI 75A)
F8	*Trametes versicolor*	HR445	Landcare Research, NZ (PB 86/097)
F9	Unknown	HR120	HortResearch, NZ
F10	Unknown	HR358	HortResearch, NZ

[a]HortResearch Culture Collection Code.

[b]Corresponding Culture Collection Code from supplier.

[c]Provided by Prof SD Aust, Biotechnology Centre, Utah State University, Logan, USA.

[d]Deposited at Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia, with accession numbers NM02/27875, NM02/27876, and NM02/27877 for Culture A, Culture B, and Culture C, respectively.

Growth substrate. Growth substrates were evaluated as individual components and/or in mixtures for supporting white-rot fungal growth, enzyme expression and ability to induce PCP degradation *in vitro*. Growth substrates consisted of apple pomace, apple pomace + untreated *Pinus radiata* sawdust (ratio1:2 on a dry weight basis), corn cobs, wheat straw, bark, composted bark, SCS (sawdust-corn meal-starch mixture as described by Leštan and Lamar (1996), and SCSL (SCS + sodium lignosulfonate) as described by Leštan et al. (1996). SCS can be pelletised easily incorporating lignosulfonate (Leštan et al., 1996). Therefore sodium lignosulfonate was added to SCS to explore if this pelletising process (Leštan et al., 1996) could be potentially applied to New Zealand native isolates by examining the suitability of SCSL as a growth substrate.

Apple pomace (ENZA Processors Ltd) was stored at -20°C until required. Upon thawing, 100 g of pomace was mixed with 1.0 - 1.5 g calcium carbonate to raise the pH from 3 to 4.5 - 5.0. All other products were stored air dry (8-12% moisture content) at room temperature 23°C. Bark, composted bark, corn cobs and straw were supplied by HortResearch. Sawdust was obtained from Tiritea Sawmill Ltd, starch and cornmeal from Davis Trading Ltd and sodium lignosulfonate from International Sales Marketing (New Zealand) Ltd. All other chemicals (analytical grade) were supplied by BDH, unless specified.

Growth substrates tested were placed in a 250 mL Erlenmeyer flask to a depth of around 20 mm. This volume (approximately 100 mL per flask) corresponded to the dry weights of 5.1 g for pomace, 11 g for pomace +sawdust, 9.9 g for corn cobs, 1.5 g for wheat straw, 8.6 g for bark, 23.1 g for composted bark, 17.6 g for SCS and 17.6 g for SCSL. The substrate mixes were adjusted to 50% gravimetric water content (Leštan et al., 1996) and autoclaved (15 min, 121°C, 120 kPa). Upon cooling, flasks were centrally inoculated with a mycelial plug (5 mm diameter) of fungal inoculum grown on PDA at 30°C for 5 days in the dark. The inoculated substrate was then incubated at 30°C in the dark. Sterile reverse osmosis water was added every 5-6 days to maintain constant moisture by weight. Three replicates were prepared for each of the tested fungal isolates.

During the 12 day incubation period in the **first experimental series**, flasks were assessed visually every 3-4 days for fungal growth using an index, with 0 = nil growth, + = sparse growth, ++ = medium growth and +++ = abundant growth. The flasks were harvested by manually disrupting the biomass-substrate composites with a sterile glass rod while still in their growth flask until the fungal biomass was evenly distributed throughout the substrate. In the first experimental series, all of the substrate mix was used for PCP degradation studies.

In the **second experimental series**, incubation was extended to 19 days. Subsamples were collected four times during incubation (9, 12, 16, 19 days) for determining the biological potential and enzyme analysis. At day 19, PCP degradation was also examined *in vitro* by measuring chloride release. In the second experiment, one isolate (F5) and two growth substrates (SCS and straw) were studied.

PCP degradation using chloride release. In the **first experimental series** all of the growth substrate-biomass mixture was used for the PCP degradation studies. A PCP

(Sigma) methanol stock solution was prepared (10000 mg/kg PCP) and diluted in a sterile glucose-water solution (9 g glucose per litre water) to reach a final concentration of 100 mg/L PCP. The PCP-glucose solution (100 mL) was added to the disrupted substrate-biomass and the mixture was shaken at 150 rpm for 24 hours at 30°C. Solids were separated from the liquid phase by centrifuging at 2500 g for 10 min. The pH of the supernatant was measured and a sample stored at 4°C for chloride analysis (Dec and Bollag, 1994) using a Buchner Digital Chloridometer Model 4-2502. Chloride readings were made in triplicate and all measurements and calculations were conducted according to the manufacturer's (Buchner) instructions. The increase in chloride levels (95% confidence) in the two subsamples was compared against the control and reported as a percentage of the maximum chloride release possible from the complete reductive dechlorination of PCP.

The considerable pH lowering (below pH 4), observed in the first experiment after the PCP-glucose solution was added prompted the use of a buffer in the PCP-glucose solution for the second experimental series. This buffer consisted of 2,2-dimethyl succinic acid (7.60 g/L) and sodium hydrogen carbonate (4.36 g/L) adjusted with dilute sulphuric acid to pH 4.3. PCP concentration was also reduced to reach a final concentration of 50 mg/L in the buffered glucose-PCP solution with a final pH of 4.5. In addition, one of the triplicate Erlenmeyer flasks was autoclaved (after collection of sub samples for biological potential and enzyme measurements) to kill the fungal biomass and provide a control for the background chloride level. On cooling, all flasks were inoculated with 50 mL of the buffered PCP-glucose solution and incubated at 30°C for 24 h. Samples were then centrifuged (2500 × *g*, 10 min), the supernatant filtered (Whatman No 2), the pH determined and chloride release measured as described above.

Determination of biological potential. The method of Leštan et al. (1996) was used to determine biological potential as measure of fungal colonization. A subsample of inoculated substrate (0.2-0.7 g wet weight) was introduced into a glass tube containing 10 mL 60 mM Na_2HPO_4 buffer (pH 8.0) and shaken vigorously. The resultant slurry was filtered through Whatmans No. 1 filter paper and centrifuged (14900 × *g*) for 10 min. To 5 mL of supernatant, 10 µL of 10 mM fluorescein diacetate (FDA, Sigma) and 10 mM dimethyl sulphoxide (DMS, BDH) solution was added to give a final concentration of 20 µM FDA/DMS. The mixture was incubated for 30 min

at 25°C and then assayed spectrophotemetrically (UV/VIS spectrophotometer model No PU8625, Philips Analytical, Cambridge Great Britain) at 490 nm. At each sampling time triplicate individual subsamples were collected and analyzed for biological potential which was subsequently expressed as the mean of the three measurements.

Enzyme assays. Approximately 1 g (wet weight) subsamples of mechanically disrupted substrate-biomass composite was extracted (Tien and Kirk, 1988) with 5 mL of 50 mM sodium malonate (pH 4.5). The centrifuged (14900 g) samples were stored up to 24 h at 4°C prior to analysis. Lignin peroxidase was determined by the peroxide-dependent oxidation of veratryl alcohol at pH 3.0 with the absorbance measured spectrophotometrically at 310 nm (Tien and Kirk, 1988). Manganese peroxidase was assayed as peroxide-dependent degradation of 2,6 dimethoxyphenol at pH 4.5 at 450 nm (Orth et al., 1993). Laccase was assayed as peroxide-independent degradation of 2,6 dimethoxyphenol at pH 4.5 at 450 nm (De Jong et al., 1994). Authentic lignin peroxidase and manganese peroxidase from *P. chrysosporium* (kindly provided by Prof. R. Farrell, University of Waikato) assayed according to Tien and Kirk (1984) served as positive controls. Triplicate sub samples were collected and analysed at each sampling time and the mean value calculated.

PCP mineralisation

Experiments in liquid culture. The New Zealand white rot fungi cultures of F4, F5, F7, F8 and F10 were examined together with the American fungus *Phanerochaete chrysosporium* (F2). Uninoculated controls were included and all treatments were made in duplicate, with some in triplicate to provide a measure of experimental variability.

Fungi were inoculated by aseptically transferring 4 mm plugs from cultures actively growing on plates of agar (20 g/L, Gibco) solidified Kirk's medium into 500 mL glass bottles (Schott Duran) containing 15 mL of sterile minimal N-deficient liquid medium (Kirk et al., 1978). A methanol solution containing around 17 kBq ^{14}C-PCP (Internationale Isotope, Munich Sp. Act. 658.6 MBq/mmol) was added by applying a methanol solution (10 µL) to a 10 mm diameter filter paper disk (Whatman No. 1), briefly driving off the solvent in an air stream, and aseptically adding the disk to the culture. Non-labelled PCP was not used in this solution as an isotopic carrier, nor were ^{14}C-PCP losses measured during the filter drying because of the very short drying time.

The bottle lid was fitted with a gas manifold, 0.45 μm pore 50 mm diameter inlet filter (Alltech), and polyurethane foam outlet filter (PUF) acting as a volatile trap (Hoff et al., 1992). Cultures were flushed every three days with a 80:20 N_2:O_2 mixture (BOC gases) and any released $^{14}CO_2$ collected in an alkaline trapping solution (Boul, 1996). Bottles were incubated static at 30°C in the dark for a total of 28 days. The bottles were sealed between flushes.

At the conclusion of the experiment volatile components trapped on the PUF plug were soxhlet extracted with acetone. The spectrum of compounds present was assessed by HPLC/scintillation counting analysis (Boul, 1996). The contents of the bottles were transferred to centrifuge tubes, spun and the pellet separated from the supernatant. ^{14}C remaining in the culture bottles was determined by washing the bottles with 10 mL acetone and measuring ^{14}C in the solution by liquid scintillation counting (LSA). The spectrum of compounds recovered in this solution was determined by HPLC and LSA. The pellet containing the fungal biomass was extracted in 10 mL acetone, and extractable ^{14}C quantified by LSA (Boul, 1996). The extracted pellet was air dried and residual ^{14}C determined by total sample combustion and LSA (Boul, 1996).

Soil microcosms. In this experiment the type of fungal strain (isolates F4, F5, F6) and soil concentration of PCP upon PCP degradation were examined. The experimental unit and conditions for the soil microcosms were identical to that for the liquid culture experiments except that the liquid medium was replaced with a mixture of garden soil and PCP polluted soil (PCP-soil). ^{14}C-PCP was added using a sand carrier (Boul, 1996). Soil PCP concentrations were adjusted using PCP-contaminated soil (approximately 30 years old) from a mill/wood treatment facility in New Zealand. The PCP-soil (contamination levels of approximately 5000 mg/kg and 265 mg/kg) was sieved (< 1 mm) and diluted with screened local soil (Temuka deep clay loam) to give PCP concentrations of approximately 50, 200, 1000 and 5000 mg/kg. The PCP contaminated soil (15 mL or approximately 16 g dry weight) then was transferred into flasks and inoculated with 20% (v/v) four-week-old fungal SCS inoculum prepared as described above. The Temuka deep clay loam pedological properties (analysed by RJ Hill Laboratories Ltd., Hamilton, New Zealand) were pH 6.2; 81% BS; 5.7% OM; 133 kg/ha N; 37 μg/mL; Olsen P; 29 μg/mL S; 3.1 cmol/kg CEC; 12.6 cmol/kg Ca; 3.39 cmol/kg Mg and 0.72 cmol/kg K.

The mineralization rate of ^{14}C-PCP at different soil PCP levels was studied in two experiments for isolates F4, F5 and F6 as described in Table 3.2. In the first experiment, sterilized fungal inoculum and sterilized soil treatments, treatments 1 and 8, respectively, served as controls. Treatments 1-4 addressed the effect of isolate; treatments 2, 5, 6 and 7 considered the effect of PCP concentration; treatments 2 and 8 looked at the effect of competing microorganisms upon PCP mineralization. Treatment 9 examined whether the original liquid culture experiments are still valid at a higher PCP concentration. Treatment 9 was prepared as described above (Experiments in liquid culture) using a higher PCP concentration. Treatments 1-4 were performed in triplicate, the remainder in duplicate. In the second soil microcosm experiment, the type of fungal strain was the only variable considered, together with killed-cell (autoclaved SCS) and sterile soil controls (treatments 1-4, 8). Since unlabelled PCP was supplied as authentic contaminated soil from a timber treatment site some variability in the PCP load, and potential interference by co-contaminants, may be expected.

Table 3.2. Experimental treatment for the soil microcosm ^{14}C-PCP mineralisation study

Treatment	Fungus	PCP (mg/kg)	Fungal Inoculum (SCS)
1	F4	200	20% (Sterilized inoculum)
2	F4	200	20%
3	F5	200	20%
4	F6	200	20%
5	F4	50	20%
6	F4	1000	20%
7	F4	5000	20%
8	F4	200	20% (Sterilized soil)
9	F4	200	Liquid culture

Results and discussion

Selection of a fungal carrier suitable for white-rot augmentation in bioremediation

Initial screening trials (**first experimental series**) based on visual estimates of growth indicated that the fungus *P. chrysosporium* outgrew the native fungi on all the substrates tested. Sparse growth was observed on straw, with somewhat better growth by all tested species on SCS and SCSL substrates. Nil to very sparse growth on bark and composted bark was observed for all fungi, with a corresponding absence of chloride release. Therefore, results on bark and composted bark are not presented. Table 3.3 summarises mean observations for fungal growth and chloride release on the other substrates tested. Whilst visual growth assessments were reproducible for all isolates on all growth substrates tested, chloride release could only be reproduced for selected isolates and selected growth substrates (Table 3.3). The presence of lignosulfonate in the formulation (SCSL versus SCS) apparently inhibited PCP dechlorination as measured by chloride release. Absence of measureable chloride release does not necessarily mean that no PCP degradation occurred, since it is possible that the different substrate mixes re-absorbed some chloride.

Table 3.3. Fungal growth on solid substrates after 12 days incubation at 30°C (PCP chloride release values (%) are given in brackets)

	Visual Growth Estimate (% chloride release from PCP)						
Isolate	Pomace	Pomace + sawdust	Sawdust	Cobs	Straw	SCS	SCSL
P.c.	+++[a] (0)	++ (0)	+ (0)	+++ (0)	+ (22)*	++ (11)*	nt
F3	++ (0)	++ (26)	0 (0)	++ (100)	nt	+ (3)	nt
F5	+ (0)	+ (22)*	0 (0)	+ (39)	+ (19)*	+ (14)*	+ (0)
F6	+ (0)	+ (0)	0 (0)	+ (30)	+ (18)*	+ (15)*	+ (0)
F9	nt	nt	nt	nt	+ (6)*	+ (21)	+ (0)
F1	nt	nt	nt	nt	nt	+ (6)	nt

P.c. = *Phanerochaete chyrososporium* (F2)

[a] +++ abundant growth; ++ medium growth; + sparse growth; 0 nil growth; nt - not tested

*reproducible growth and chloride release in subsequent screening experiments

Of the native isolates tested, F3 was observed to have the most biomass on pomace, pomace/sawdust and corn cobs, together with the highest PCP chloride release (26%) when grown on pomace/sawdust. However, the apparently high chloride release values observed for biomass grown on corn cobs is open to question as the chloride level of the dry cobs was 0.3%, which possibly contributed to a high chloride background in the control. In subsequent experiments with cobs and straw, an apparently high chloride level in the controls gave rise to inconsistent, negative chloride levels in the PCP supernatants. This may be due to soluble substances from these two substrates interfering with the electrochemistry of the Chloridometer.

The pH of the PCP solutions after 24 h had dropped in all flasks from an initial pH of 7.7 to values of ≤ 6. The lower pH values were observed even when the PCP solution had no added glucose, indicating that the pH lowering was due to metabolites produced by the fungi during growth. The pH of the PCP solutions of the SCS substrate ranged from 3.1 to 3.8, the lowest values of all the substrates investigated. In subsequent trials testing the effect of pH on PCP chloride release (data not presented) chloride release was reduced in the lower pH ranges. These findings are in agreement with research by Lamar et al. (1990) who suggested that low pH values may be sub-optimal for PCP degradation.

The results in Table 3.3 indicated that native white-rot isolates grown on waste materials dechlorinate PCP and therefore may have potential to mineralize PCP. The results also indicated that colonisation of different growth substrates is not only isolate dependent, but also substrate dependent. The results further implied that colonisation may not relate to degradation potential. Therefore one isolate (F5) was selected in the second experiment to explore in more detail the relationship between growth substrate colonisation (biological potential), enzyme activity and PCP dechlorination. Straw and SCS were chosen as substrates, because of their availability and relatively low cost in New Zealand.

Biological potential, enzyme activity and PCP dechlorination. The results for the **second experimental series** investigating the relationship between substrate colonisation, enzyme profile and PCP degradation were highly reproducible and are presented in Table 3.4. Biological potential increased during incubation. In contrast, laccase activity decreased during incubation. Laccase was the only enzyme detected.

Table 3.4. Biological potential, laccase activity and chloride release measured on two growth substrates during 19 days incubation at 30°C using isolate F5

	Biological Potential[a]				**Laccase (nmole/min/g)**[b]				**PCP chloride release (%)**			
Time	SCS		Straw		SCS		Straw		SCS		Straw	
(days)	E1[c]	E2	E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
9	<1	16	4	136	71	88	227	182	nt	nt	nt	nt
12	24	39	44	204	53	109	236	331	nt	nt	nt	nt
16	40	69	115	237	11	49	109	434	nt	nt	nt	nt
19	45	111	175	372	20	162	132	730	19.5	nt	38	nt

[a] µM fluorescein developed during 30 min incubation/g substrate (dry weight).

[b] based on dry weight.

[c] E1 - experiment 1, E2 - experiment 2, nt - not tested.

Biological potential, laccase production and chloride release were higher on straw than on SCS. The absence of lignin peroxidase and managanese peroxidase may be expected in an isolate of *Trametes versicolor*, as this species is well known to predominantly produce laccase (Pointing, 2001; Gianfreda et al., 1999).

Biological potential of isolate F5 was close to its maximum when grown on SCS at approximately 19 days, but was still increasing in the case of straw. The corn meal and starch components of the SCS formulation improved growth rate compared with sawdust alone. This is in agreement with the findings of Leštan et al. (1996) who reported that inoculum biological potential was greatly influenced by substrate formulation and structure, and temperature. They also found that biological potential and substrate type influenced the ability of white-rot fungi to tolerate PCP and induce ligninase activities.

PCP Mineralisation

In liquid culture, visible mycelial growth occurred for all isolates. *P. chrysosporium* grew faster than the native fungi, based on a visual assessment of mycelial bulk. F4 and F5 also grew relatively rapidly but the remaining fungi grew

slower. The distribution of ^{14}C originally added as PCP after 28 days incubation is shown in Table 3.5. In the uninoculated controls the majority of the isotope was recovered from the volatile trap, with most of the remainder in the filtered medium. No significant ^{14}C was recovered from the CO_2 trap. The pattern observed in microcosms containing *P. chrysosporium* was generally similar. In microcosms containing NZ strains, PCP degradation to CO_2 occurred between 1 and 20% (means 2-16.8%) conversion. Strains F4, F5 and F7 converting greater than 10% during the experiment. It is noteworthy, that the isolates F4 and F5 with the largest, cumulative $^{14}CO_2$ release also had the lowest mass recovery rates. This suggests that there might have been a small leak in the CO_2 traps. However, variation in $^{14}CO_2$ evolution between strain replicates was reasonable as demonstrated for F4 (Figure 3.1) - the isolate or treatment with the largest variation between replicates. The figure clearly shows that the rate of mineralization or $^{14}CO_2$ release is similar between replicates. Variation between replicates may be due to slightly different mycelial growth rates, level of enzyme activities and/or CO_2 trap efficacy – if indeed some leakage occurred.

Table 3.5. Distribution of ^{14}C recovered from various fractions in the liquid culture experiment. Numbers are percent of radioactivity initially added

Fractions	**Fungal Isolate**						
	F2	F4	F5	F7	F8	F10	Control
Pellet extract (total)[a]	3.3	5.6	0.6	5.8	3.4	0.1	1.7
Volatile	90.3	29.3	26.2	38.4	68.5	78.9	95.3
Culture filtrate	1.8	29.8	25.5	29.2	13.0	10.9	6.9
CO_2 (cumulative total)	0.3	16.8	14.8	10.6	5.5	2.0	0.2
Pellet combustion	0.5	4.3	7.9	5.6	3.0	5.2	0.1
TOTAL[b]	96.5	88.4	83.5	92.3	94.5	103.4	107.1

[a] Pellet consists of the filter paper disc used to inoculate the microcosm with PCP.

[b]Total, additionally contains minor fractions, such as equipment washings that are processing artefacts. They have been included in the total to demonstrate the efficiency of the techniques even though they do not represent any meaningful isotopic fate.

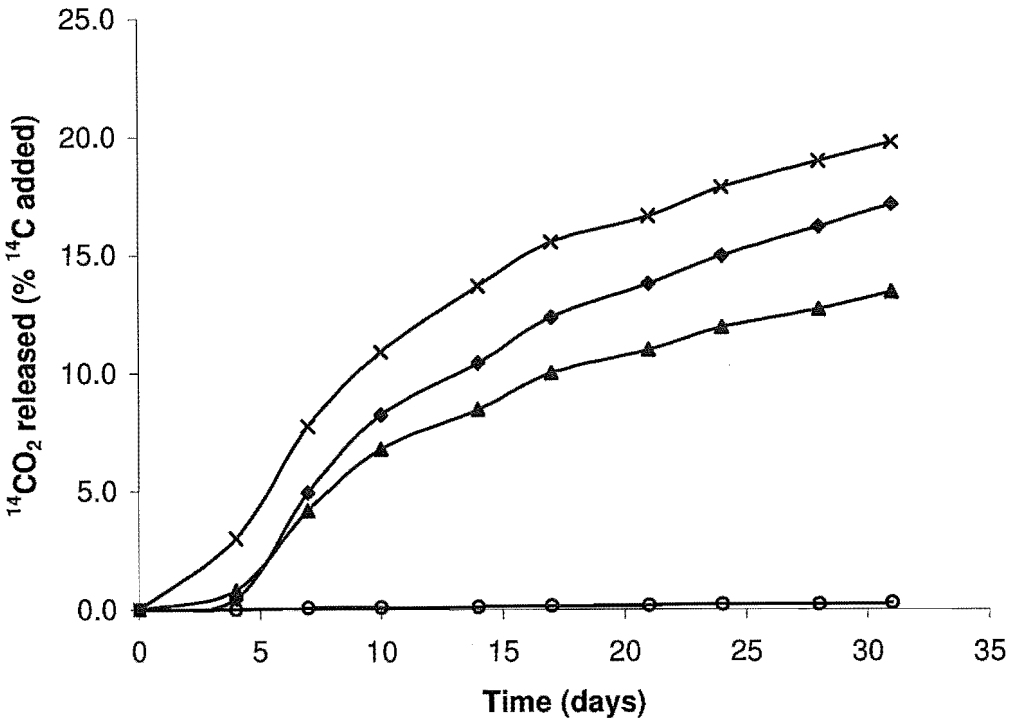

Figure 3.1. Release of $^{14}CO_2$ from ^{14}C-PCP by strain F4 in liquid culture. Lines represent cumulative $^{14}CO_2$ independent microcosms. Open circles are mean data for the uninoculated control (pooled standard error of the mean SE = 0.05).

The majority of ^{14}C was recovered from the volatile trap averaging 90 and 95% in the control and *P. chrysosporium* (F2) cultures with 30 and 26% in the F4 and F5 cultures, respectively. HPLC analysis of the trapped volatiles showed the volatilized residues were almost entirely PCP in the native strain cultures, and a trace of pentachloroanisole (PCA). In comparison, PCA comprised around 75% of the residues volatilized by *P. chrysosporium*. The filtrate contained low ^{14}C in control (7%) and *P. chrysosporium* (2%) bottles. In the native cultures, 11-30% of added ^{14}C was recoverable in this fraction. Reverse phase HPLC analysis of the filtrate demonstrated the label was associated with very polar compounds and there was virtually no ^{14}C-PCP or ^{14}C-PCA. Acetone extraction of the biomass released 3-7% of the added ^{14}C, while a further 3-8% remained unextractable (determined by combustion and scintillation counting of liberated $^{14}CO_2$). Negligible amounts of ^{14}C were associated with these fractions in control and *P. chrysosporium* inoculated bottles.

It is clear that, under the culture conditions examined, the New Zealand white rot fungi strains were able to degrade PCP to aqueous soluble metabolites and CO_2. *P.*

chrysosporium, which has been shown to degrade PCP in other studies (Mileski et al., 1988), did not do so under the conditions of these experiments. Furthermore the New Zealand strains did not produce appreciable levels of the undesirable product pentachloroanisole which is considered an intermediate metabolite in PCP degradation by *P. chrysoporium* (Leštan et al., 1996). The total mass loading of PCP in these experiments was determined by the requirement for practical levels of ^{14}C, and was thus very low (< 1 µg/mL). These experiments demonstrate the capacity of New Zealand fungal strains to degrade and mineralize PCP. The conditions are, however, quite different to those that might be expected in a field remediation situation, both with respect to PCP concentration and fungal growth conditions.

Results of the two soil microcosm experiments are given in Table 3.6 and Table 3.7. In the soil microcosm experiments, combustion analysis of the extracted soils was not performed. The relatively low total recoveries of ^{14}C can most likely be attributed to PCP forming non-extractable residues in the soil and inoculum material. However, this was not specifically measured.

Table 3.6. Distribution of ^{14}C recovered from various fractions in the first soil microcosm experiment. Numbers are percent of radioactivity initially added

Treatment	Fungus	PCP (mg/kg)	Inoculum (v/v)	CO_2 (%)	Volatile (%)	Extractable (%)	Aqueous phase (%)	Total (%)
1	F4	200	20% (Control[a])	6	1	33	na	40
2	F4	200	20%	40	1	12	2	53
3	F5	200	20%	39	1	14	2	54
4	F6	200	20%	41	1	8	3	53
5	F4	50	20%	38	2	3	6	50
6	F4	1000	20%	4	0	27	1	32
7	F4	5000	20%	2	0	36	0	38
8	F4	200	20% (Control[b])	3	2	25	3	33
9	F4	200	Liquid culture	2	64	0	0	66

[a] inoculum sterilized by autoclaving

[b] soil sterilized by autoclaving

na - data not available

Table 3.7. Distribution of ^{14}C recovered from various fractions in the second soil microcosm experiment. Numbers are percent of radioactivity initially added

Treatment	Fungus	PCP (mg/kg)	Inoculum (v/v)	CO_2 (%)	Volatile (%)	Extractable (%)	Aqueous phase (%)	Total (%)
1	F4	200	20% (Control[a])	3.3	1.2	30.1	6.5	41
2	F4	200	20%	22.9	0.7	13.6	4.7	42
3	F5	200	20%	14.0	1.0	20.3	4.5	40
4	F6	200	20%	33.9	0.7	6.2	3.4	44
5	F4	200	20% (Control[b])	11.0	1.3	17.6	5.0	35

[a] inoculum sterilized by autoclaving

[b] soil sterilized by autoclaving

Radiotracer methods were used as they allow (a) determination of mineralization to $^{14}CO_2$, (b) determination of mass balances, (c) determination of unextractable or bound residues and (d) confirmation that detected residues arose from the added contaminant although this was not determined in all the described experiments. It is clear that the fungi were able to significantly degrade PCP in unsterilized soil at concentrations up to 200 mg/kg. At nominal PCP concentrations of 200 mg/kg, there was little difference between the strains in the first experiment, though they were more variable in the second. Nevertheless, for these treatments, a significant amount of PCP was mineralized over the duration of the experiments.

It is probable there was very little degradation of PCP at levels of 1000 and 5000 mg/kg. The data may be misleading, as the proportion of total PCP due to ^{14}C-PCP in these treatments is less than in 200 mg/kg treatments. This should not affect the result obtained as it is assumed the same proportion of labeled and unlabelled PCP is degraded. However, it is likely that freshly added, unaged, labeled PCP is more readily degraded than the aged native PCP in the soil (Northcott and Jones, 2001). Therefore, a greater proportion of labelled PCP could be degraded. Thus when the degradation of ^{14}C-PCP was converted to a total PCP mass basis, the mineralization occurring in the

1000 and 5000 mg/kg treatments appears significant. However, the amount of ^{14}C found in the CO_2 trap in both these treatments is less than the autoclaved control. Therefore the initial assumption is probably invalid and it could be concluded that the fungi did not degrade 1000 and 5000 mg/kg PCP to a level significantly different from that determined for the control sample. Nevertheless degradation at PCP concentrations greater than 200 mg/kg cannot be ruled out from the data presented.

Dilution of polluted PCP soil with non-contaminated local soil introduced different microbial populations and soil characteristics to the degradation reactor and therefore potentially influenced the fate of PCP in the microcosms. It is clear from the sterilized inoculum data that viable white-rot fungus was necessary for significant PCP degradation. While there was potential for native fungi present in the sampled soil to colonize the added substrate and metabolize PCP, this was not observed to any great extent over the timeframe of the experiments. Interestingly, it appeared that the fungi were not able to degrade PCP in soil in the absence of other organisms as PCP degradation in the presence of viable F4, but in sterilized soil it was negligible. An alternative interpretation may be that autoclaving has caused some alteration within the soil that inhibits the degradation process. This could conceivably be due to decreased availability of the PCP due to tighter binding to the soil or release of inhibitory compounds. Another explanation may be that the fungi metabolize components of the soil and/or growth substrate that in turn initiate microbial degradation of PCP, or the fungi metabolize PCP to a major intermediate metabolite that is subsequently degraded by soil microbes.

It has been argued that bioaugmentation is a poor remediation strategy in that organisms with degradation capacity may not compete well with acclimated organisms already present or that in doing so degradation capacity is affected. The results of these experiments, if not artefacts of autoclaving, suggest that these arguments do not hold for isolate F4. The requirements for other organisms and/or nutrients for biodegradation were not examined. Results from pure liquid culture with a very low PCP concentration (<1 μg/mL) clearly show that the fungi are able to carry out complete degradation. In contrast, PCP supplied to liquid cultures at rates comparable to the soil microcosms (200 mg/kg) was not degraded. This suggests that PCP at this level is toxic or inhibitory under these conditions. Clearly this is not so in the presence of soil, and it is possible that binding of PCP by soil components reduces the immediate bioavailability

of the compound allowing the fungi to grow in the soil environment and concomitantly and/or subsequently degrade the pollutant.

The very large volatile fraction measured in the liquid culture experiments, may be an artefact of the experimental design. The PUF volatile trap is within the culture bottle. It is possible that mass-transfer between solid phase PCP, the solution phase, vapor phase, and the trapped volatiles has occurred. The PUF trap has a high affinity for PCP therefore the equilibrium lies strongly in that direction. Passive sampling by PUF plugs usually results in non-reversible trapping. The high vapor pressure and Henry's law constant for PCP from the liquid to gas phase support rapid volatilization and trapping of PCP. An alternative explanation is that trapping by the PUF is slow and degradation occurs prior to the PCP reaching the trap. However, this was not supported by the HPLC analysis which showed the activity in the volatile trap was almost all PCP, with traces of PCA. Therefore, the rate of PCP mass-transfer to the PUF plug was much greater than the rate of PCP degradation in the liquid phase. Whatever the cause of the high volatile release in liquid culture it was not observed in the soil microcosms. It is probable that soil and added organic matter retain PCP sufficiently to significantly reduce volatilization. This is of importance for any field remediation solution as the release of volatile contaminants is generally undesirable.

This study clearly indicates that New Zealand native *T. versicolor* isolates showed potential for remediation of PCP polluted soil sites stemming from past industry practices. This is in agreement with findings by Tuomela et al. (1999). Their research showed that *T. versicolor* mineralised PCP in soil. However, scale-up and the effect of pollutant concentration on PCP degradation need to be further clarified to determine engineering requirements and the concentration threshold, where pollutant concentration impedes degradation processes.

Laccase was the only enzyme detected. *T. versicolor* was able to mineralise 21% ^{14}C-PCP while secreting laccase but no peroxidases in liquid culture. Previous work has shown that there is no correlation between laccase secretion and PCP degradation (Ricotta et al., 1996). To date, there is no conclusive evidence to link laccase activity to PCP-degrading ability of white rot fungi in soil systems (Pointing, 2001), but treatment of PCP polluted soils with *T. versicolor* is possible (Tuomela et al., 1999). The PCP degradation pathway by New Zealand *T. versicolor* is not yet fully understood and further research on the bio-chemical processes involved in *T. versicolor* PCP degradation is required.

SCS and wheat straw have been demonstrated as suitable carriers for *T. versicolor* inocula for augmentation in bioremediation. In New Zealand, SCS is preferable when considering the practical aspects of scaling-up white rot fungi, due to substrate availability and cost of the carriers. Pine sawdust is freely available at little to no cost, compared to the relatively small wheat production and expense of the straw. Further research is required to investigate isolate specific requirements, determine optimum conditions for fungal colonisation of a carrier for bioremediation, scale-up of inoculum production and the nutritional requirements inducing bioremediation activity

Acknowledgements

This work was funded by the Foundation for Science, Research and Technology. We are grateful to Dr Roberta Farrell, University of Waikato, for provision of enzymes. Our thanks to Prof Steven Aust, Utah State University, and staff at Forest Research and Landcare Research for provision of fungal isolates. Thanks to Dr Adrian Spiers, Dr Ann Slade and Ms April Shannon for collection and identification of HortResearch isolates and maintenance of the culture collection. Thanks also to Dr John Christeller for verifying the presence (or absence) of the enzymes studied.

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Chapter 4

Detection of *Trametes versicolor* isolates in soil by PCR

Foreword

Chapter 4 explores the use of molecular tracking methods for monitoring the survival and growth of white-rot fungi in soil. Originally, the molecular biology PhD component was intended to play a more significant part in the thesis. However, with the University of Canterbury supervisor leaving New Zealand (and no suitable advisor being available) and the employment of a molecular biologist by HortResearch, to fast-track the work, the emphasis of the molecular biological tracking tool development for the PhD project was re-evaluated. The work presented here was carried out solely by M. Walter over a 4-month part-time period. PCR descriptions, template lay-outs and corresponding photos of the gels are presented in Appendix to Chapter 4. That section also contains a copy of the report obtained from automated sequencing. A modified version of the chapter below, including additional research by Siva Sivakumaran, will be submitted for publication by Methods in Environmental Microbiology.

Modified publication by

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Modified version to be submitted in conjunction with subsequent work by S. Sivakumaran to Methods in Environmental Microbiology

Abstract

DNA was obtained from soil from three *Trametes versicolor* isolates using bead beat extraction combined with PVPP (polyvinylpolypyrrolidone) and sephadex-G75 extract clean-up and subsequent PCR amplification using ITS-1 and ITS-2 primers. Detection limit of mycelium was dependent on isolate, soil type and mycelium type (fresh versus freeze-dried). Nested PCR greatly enhanced *T. versicolor* detection compared to single PCR amplifications. DNA from fresh mycelium was more readily amplified (approximately 10 fold) than freeze-dried mycelium (based on mycelial dry weights). White-rot fungal DNA was readily extracted and detected using ITS primers from both sterile and non-sterile soils. Soil type greatly influenced fungal DNA detection limits.
Keywords: PCR, ITS-1, ITS-2, *Trametes versicolor*, detection in soil, detection limits, soil type

Introduction

White rot fungi *Trametes versicolor* (Linnaeus: Fries) Pilat (Fam. Polyporaceae) have the potential for field bioremediation because of their ability to degrade a variety of xenobitoic chemicals via free radical mechanism mediated by extracellualr peroxidases. Reliable methods for quantifiably detecting these organisms in field biopiles are needed if bioremediation is to be monitored and optimised.

Detecting microorganisms by PCR amplification of DNA extracted from environmental samples is much more rapid than culture techniques which require recovery and growth of active organisms (Johnston and Aust, 1994; Sivakumaran et al., 1997). Extraction of total DNA followed by PCR amplification has been shown to to be a senstive method for detecting specific bacteria in soil samples (Hilger and Myrold, 1991; Ogram et al., 1988; Picard et al., 1992; Steffan and Atlas, 1988; Tebbe and Vahjen 1993; Tsai and Olson 1992). Such an approach developed for bacterial DNA can also be used to detect fungal DNA (Tebbe and Vahjen, 1993). Eukaryotic ribosomal DNA genes have high copy numbers as well as interspersed conserved and variable DNA sequences (White et al., 1990), making them ideal targets for species identification by PCR amplification coupled to restriction enzyme analysis. The internal transcribed spacer (ITS) region of fungal ribosomal DNA has been used for resolving species of other genera (Gaskell et al., 1992; Hibbet and Vilgayls, 1991). White-rot

fungi do not occur naturally in soils, but are able to colonise contaminated soils if provided with a nutrient reserve (Leštan and Lamar, 1996). This approach could be used to indicate the presence or absence of the bioaugumented white rot fungi in soil. In this study, the use of polymerase chain reaction (PCR) to detect *T. versicolor* mycelium in soils was investigated.

Materials and methods

Soils and microorganisms

Three soils were collected to a depth of 30 cm from New Zealand. The soils were a Temuka deep clay loam, Bealey silt loam and sand (fluvial raw) as described by Boyd-Wilson et al. (2005 – Chapter 6). Soils were sieved (< 4 mm), air-dried and stored in plastic bags at 4°C. Soils were used either sterile (by autoclaving twice for 1 hour at 121°C, 10 kPa on two successive days) or non-sterile as outlined below.

Three isolates of *T. versicolor*, HR131, HR154 and HR160 (HortResearch Culture Collection, Lincoln), capable of degrading PCP (Walter et al., 2003) were maintained as mycelial plugs in 7 mL bijou bottles (Samco Laboratories) of sterile distilled water at room temperature. Fungal inoculum was either used fresh or freeze-dried and augmented to the soils as described below. Fresh mycelium was produced by transferring mycelial plugs (6 mm diameter) from the bijou bottles onto malt extract agar (MEA, Merck) and incubating at 30°C for 6 days in the dark. Freeze-dried mycelium was produced by inoculating 50 mL sterile malt extract broth (Merck) with a 6 mm mycelial plug in a 250 mL earlenmeyer flask. Flasks were incubated stationary at 30°C in the dark for 14 days, mycelium harvested (by decanting the broth), rinsed twice with sterile distilled water and freeze-dried (-80°C) in sterile universals. Mycelial weight was determined before and after the freeze-drying.

DNA extraction and PCR

Soil-DNA was extracted using the bead-beat extraction method and extract purification as described by Cullen and Hirsch (1998). Primers (Gibco-BRL) were ITS1 (TCC GTA GGT GAA CCT GCGG) and ITS2 (GCT GCG TTC TTC ATC GATGC). For the *T. versicolor* isolates, PCR and nested PCR was performed using 1 µL DNA and PCR template, respectively, 41 µL sterile double distilled water (DDW), 10 µL 10x

Taq reaction buffer (Qiagen), 8 µL of 2 mM dNTPs master mix (Gibco-BRL, final concentration 160 µM), 22.5 µL ITS-1 and 17 µL ITS-2 (both with a final concentration of 100 pmol) and 0.5 µL of *Taq* polymerase (2 units, Qiagen) resulting in a total of 100 µL reaction mixture. This was overlayed with 30 µL of sterile mineral oil. Each tube was mixed by a quick pulse in a microcentrifuge and placed in the thermal cycler (Corbett Research FTS – 320 Thermal Sequencer). The PCR conditions consisted of 26 cycles of 94°C for 1 min, 42°C for 2 min and 74°C for 3 min followed by a final extension at 4°C for 5-10 min. PCR amplicons (12.5 µL mixed with 2 µL bromophenol blue gel loading dye) were separated by electrophoresis through 2% agarose gels using Tris Acetate gel buffer at 90 volts for 1 h. A 100 bp and/or 1000 bp DNA ladder (Gibco-BRL) were used as a molecular weight marker. Gels were stained for 20 min with 1 x TAE buffer containing 0.5 µg/mL ethidium bromide. All PCR reactions were run with a negative control (substituting the DNA or PCR template with 1 µL DDW) and a positive control containing purified fungal DNA (1 µL, approximately 5-10 ng) from freeze-dried or fresh mycelium. Purified DNA templates from the three *T. versicolor* isolates were also sequenced automatically (Auckland University).

Detection of T. versicolor *in soil*

Five experiments were conducted. In Experiment 1, the soil extraction and PCR protocols were tested for isolate HR154. In Experiment 2, the detection limit of augmented freeze-dried mycelium from soil was determined for the three strains of *T. versicolor* for the three soils. Experiment 3 was a modified repeat experiment of Experiment 2 using different amounts of freeze-dried mycelium and in Experiment 4 the detection limits of augmented fresh *T. versicolor* mycelium from soils was investigated in comparison to freeze-dried mycelium. Experiment 5 further determined and verified detection limits of fresh mycelium added to soil. In this final experiment mycelium was also added to soil on a sawdust-corn meal-starch carrier (SCS; as described by Leštan et al., 1996) suitable for augmentation of white-rot fungi to polluted soil. Table 1 details the parameters studied in Experiments 1-5. For the experiments, 0.5 g air-dried soil (1 g for Experiment 1) was weighed into sterile bead beating vials (2 mL), fungal mycelium added (see concentrations in Table 1) and after 1 h incubation at room temperature DNA extracted (Cullen and Hirsh, 1998).

Table 4.1. Experimental parameters studied in Experiments 1 to 5

	Soil type[a]	Sterile	*T. versicolor* isolates	Mycelium added (mg) Freeze-dried	Fresh
Exp 1	Temuka	Yes/No	HR154	0; 60	-
	Sand	Yes/No	HR154	0; 60	-
Exp 2	Bealey	No	HR131	0; 2.5; 5; 10; 20; 30	-
	Bealey	No	HR154 & HR160	0; 30	-
	Temuka	No	HR131	0; 2.5; 5; 10; 20; 30	-
	Temuka	No	HR154 & HR160	0; 30	-
	Sand	No	HR131	0; 2.5; 5; 10; 20; 30	-
	Sand	No	HR154 & HR160	0; 30	-
Exp 3	Bealey	No	HR131	0; 10; 30; 60; 120	-
	Bealey	No	HR154 & HR160	0; 60	-
	Temuka	No	HR131	0; 10; 20; 30	-
	Temuka	No	HR154 & HR160	0; 60	-
	Sand	No	HR131	0; 10; 20; 30; 60; 120	-
	Sand	No	HR154 & HR160	0; 60	-
Exp 4	Nil	-	HR131	60	600
	Nil	-	HR154 & HR160	60	600
	Bealey	No	HR131	0; 60	60; 300; 600
	Bealey	No	HR154 & HR160	60	600
	Temuka	No	HR131	0; 60	60; 300; 600
	Temuka	No	HR154 & HR160	60	600
	Sand	No	HR131	0; 60	60; 300; 600
	Sand	No	HR154 & HR160	60	600
Exp 5	Temuka	No	HR131	-	600 SCS[b]
	Temuka	No	HR154 & HR160	-	600 SCS[b]
	Sand	No	HR131	-	600 SCS[b]
	Sand	No	HR154 & HR160	-	600 SCS[b]
	Bealey	No	HR131	-	0; 6; 30; 60; 300; 600
	Bealey	No	HR154 & HR160	-	60
	Temuka	No	HR131	-	0; 6; 30; 60; 300; 600
	Temuka	No	HR154 & HR160	-	60
	Sand	No	HR131	-	0; 6; 30; 60
	Sand	No	HR154 & HR160	-	60

[a] Air dry soil weights were 0.5 g except for Experiment 1, with soil weights of 1 g

[b] SCS = fungal growth substrate based on a sawdust-corn meal-starch mixture (Leštan et al., 1996)

Results and discussion

The weight of the freeze-dried mycelium was approximately 10% of the fresh mycelial weight. The three *T. versicolor* isolates all showed a band of approximately 300 bp. Automated sequence results for HR131 and HR154 are shown in Appendix to Chapter 4.

In Experiment 1, the 60 mg freeze-dry mycelium of the selected *T. versicolor* isolate HR154 was detectable in the two soils studied, both sterile and non-sterile, using nested PCR. No bands were shown on the soil only extractions where no mycelium had been added. There was no difference in the band intensity after gel electrophoresis between sterile and non-sterile soils. Thus all further experiments were conducted using non-sterile soils.

In Experiment 2, isolate HR131 could be detected in the Sand and Temuka soils at mycelial augmentation of 30, 20 and 5 mg. None of the other fungi could be detected. This implies that there was a soil effect on fungal detection limit as well as an isolate effect. This initial analysis also suggests that 4-6% (w/w) freeze-dried mycelium is required for detection using PCR gel-electrophoresis.

In Experiment 3, after the first PCR amplification only isolate HR131 (30 mg) in Sand was detectable. After the nested PCR, HR131 was detected at all concentrations in all soils. Isolates HR154 and HR160 were not detected at 30 mg but were detected at 60 mg in all three soils using nested PCR (Figure 4.1). The results of Experiment 3 verify findings in Experiment 2 and further indicate that fungal detection is isolate and soil type dependent. It is noteworthy that compared to Experiment 1 soil volumes in Experiments 2 and 3 were halved, but the final template was still recovered with the same extraction volume (200 µl). This may explain the lack of detection of freeze-dried mycelium at 30 mg using 0.5 g soil volumes, compared to 60 mg freeze-dried mycelium in 1 g soil.

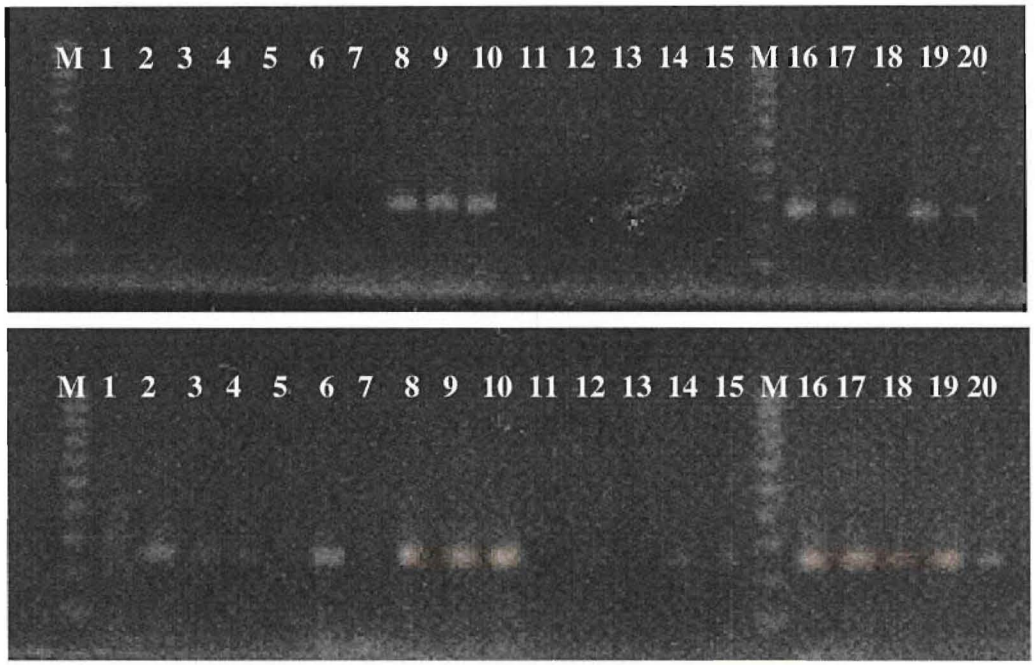

Figure 4.1. Gel showing sensitivity and detection by PCR of ITS DNA amplified with ITS2 and ITS3 primers for single PCR (top) and nested PCR (bottom) for *T. versicolor* DNA extracted from three different isolates and three different soils in Experiment 3. Treatments were:

Lane	Treatment code	Fungal isolate	Freeze-dried mycelium added	Soil
M	100 bp Marker	-	-	-
1	150	Nil	0 mg	Temuka
2	151	HR131	10 mg	Temuka
3	152	HR131	20 mg	Temuka
4	153	HR131	30 mg	Temuka
5	154	HR131	60 mg	Temuka
6	155	HR131	120 mg	Temuka
7	156	Nil	0 mg	Sand
8	157	HR131	10 mg	Sand
9	158	HR131	20 mg	Sand
10	159	HR131	30 mg	Sand
11	160	Nil	0 mg	Bealey
12	161	HR131	30 mg	Bealey
13	162	HR131	60 mg	Bealey
14	163	HR131	120 mg	Bealey
15	164	HR154	60 mg	Temuka
16	165	HR154	60 mg	Sand
17	166	HR154	60 mg	Bealey
18	167	HR160	60 mg	Temuka
19	168	HR160	60 mg	Sand
20	169	HR160	60 mg	Bealey

In Experiment 4, the detection of fresh mycelium was generally greater than equal amounts (dry weight basis) of freeze-dried mycelium for both single and nested PCR runs. Nested PCR was required to detect DNA from freeze-dried mycelium, whereas fresh mycelium was readily detected using single PCR. Similarly, in soil, fresh mycelium was more readily detected than freeze-dried mycelium (Figure 4.2). The

detection limit of fresh mycelium was as little as 60 mg/0.5 g air dried soil, as also confirmed in Experiment 5.

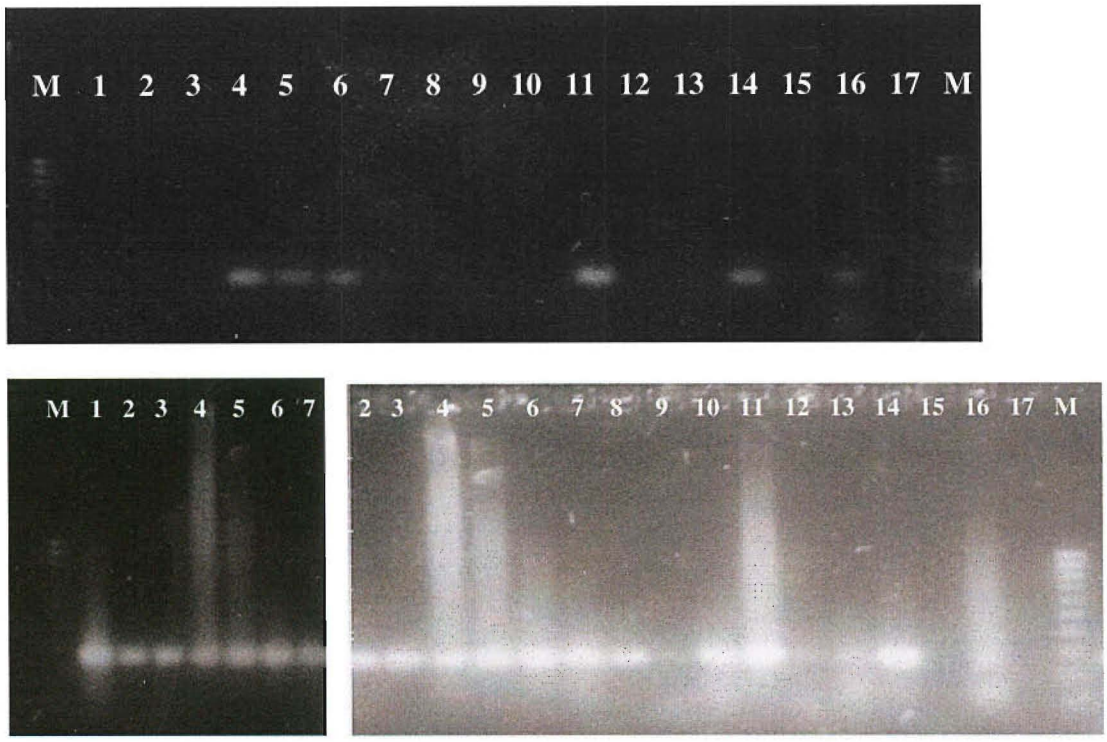

Figure 4.2. Gel showing sensitivity and detection by PCR of ITS DNA amplified with ITS2 and ITS3 primers for single PCR (top) and nested PCR (bottom) for fresh mycelium from *T. versicolor* isolates added to three different soils in Experiment 4. Treatments were:

Lane	Treatment code	Fungal isolate	Fresh mycelium added	Soil
M	100 bp Marker	-	-	-
1	190	HR131	600 mg	Temuka
2	191	HR131	300 mg	Temuka
3	192	HR131	60 mg	Temuka
4	193	HR131	600 mg	Sand
5	194	HR131	300 mg	Sand
6	195	HR131	60 mg	Sand
7	196	HR131	600 mg	Bealey
8	197	HR131	300 mg	Bealey
9	198	HR131	60 mg	Bealey
10	199	HR154	600 mg	Temuka
11	200	HR154	600 mg	Sand
12	201	HR154	600 mg	Bealey
13	202	HR160	600 mg	Temuka
14	203	HR160	600 mg	Sand
15	204	HR160	600 mg	Bealey
16	Postive control	HR131	PCR with purified DNA	-
17	Water control	-	-	-

Experiment 5 showed that *T. versioclor* DNA was readily extracted from soil when the fungus was augmented using the growth substrate SCS. The Experiment also validated that the detection limit of fresh mycelium was 60 mg per 0.5 g soil

irrespective of soil type. However, detection in sand still occurred at 6 mg/0.5 g sand using nested PCR.

The preliminary research presented here is in agreement with findings by Johnston and Aust (1996). They reported that amplification of the ITS region was more sensitive than amplification of ligninase H8 DNA. They then concluded that ITS is a potentially more easily detectable target for the white-rot fungus *Phanerochate chrysosporium* Burds. Johnston and Aust (1996) also found that detection of white-rot fungal DNA in soil was not as sensitive as for pure cultures. This is readily explained by the interference of humic materials (Tebbe and Vahjen, 1993) and clay particles (Ogram et al., 1987). Humic material co-purifies with DNA during extraction and clay binds DNA adsorptively reducing the yield of DNA extracted from soils. These interferences by humic materials and clay also explain the effect of soil type on detection limits (as shown in this study), suggesting that DNA extractions need to be adjusted/modified for different soils.

Conclusion

Bead-beat extraction and PCR amplification allow the detection of *T. versicolor* in soil. However, for *Trametes* isolates, laccase extraction from soil (Lang et al., 1997) may be a cheaper and faster detection approach than using PCR. The latter was found more time-consuming, more costly and less sensitive than laccase extraction and detection with a UV-vis spectrophotometer as shown by the student project from Schmidt (2003).

Acknowledgements

Thanks to the Foundation for Research, Science and Technology for funding. Thanks to Dr John Klena, Megan Mahey and the postgraduate students in the molecular biological laboratory for support and help.

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Chapter 5

The effect of isolate and soil parameters on soil colonisation by *Trametes versicolor*

Foreword

Chapter 5 studied various soil factors (e.g. water content, pH, temperature) and their influence on soil colonisation by *Trametes versicolor*. Chapters 5 and 6 were studied more or less hand-in-hand, with Chapter 5 focusing predominantly on soil parameters and Chapter 6 addressing the effect of different soil types on fungal on fungal growth in soil. The experiments were designed and carried out by M. Walter with technical assistance from K.S.H. Boyd-Wilson and J.H. Perry. The size of the experiments (fully factorial, up to five factors studied) required help. The design of the experiments and the statistical analysis was done by M. Walter, with guidance from C.M. Frampton, formerly of Lincoln University. ANOVA tables are presented in detail in Appendix to Chapter 5. The student also is the senior author of the modified publication (excluding the 'Additional information' section), which was submitted to Jounral of Environmental Management in June 2004 and revised in December 2004.

Modified publication by

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The effect of isolate and soil parameters on soil colonistation by *Trametes versicolor*. Submitted to Journal of Environmental Management June 2004, revised December 2004, accepted March 2005.

Abstract

New Zealand has a large number of sites contaminated by persistent chemicals that affect land use and threaten local and regional ecosystems. Using isolates of indigenous white-rot fungi (*Trametes versicolor*) as bioremediation agents, the clean-up of the timber-treatment chemical pentachlorophenol (PCP) was used as a model system. The effects of soil and biopile parameters (strain variation, water requirements, temperature, fungal growth substrate (SCS) and pollutant concentration) on fungal growth and survival were determined to establish the most effective conditions required for New Zealand white-rot bioremediation in New Zealand soils. Soil colonisation was significantly dependent on isolate and soil type, ranging from sparse to complete colonisation for the 3 different *Trametes versicolor* isolates studied, with a significant interaction between soil type and fungal isolate. Soil colonisation was also affected by augmentation with SCS. The concentration of SCS was less important than the presence of SCS (>25%). Other parameters that significantly affected fungal growth were temperature, pH, soil moisture level and pollutant concentration. For successful bioremediation in the field good colonisation of the polluted soil is desirable and the rate-limiting factors for colonisation and bioremediation need to be identified.
Keywords: *Trametes versicolor*, white-rot fungi, soil colonisation, pH, moisture, growth substrate concentration, PCP, pentachlorophenol.

Introduction

Pentachlorophenol (PCP) is one of the worst environmental pollutants (Tuomela et al., 1999). Bioremediation of PCP has gained more attention than biodegradation of other pollutants (Pointing, 2001). PCP can be degraded by bacteria and by a wide range of fungi as reviewed by McAllister et al. (1996). Most fungal studies have focused on the lignin-degrading white-rot fungi (McAllister et al., 1996).

Bioremediation using white-rot fungi requires augmentation of the organism using a suitable growth substrate (Leštan et al., 1996). In general, good fungal soil colonisation is advantageous for fungal biodegradation (Boyle, 1995). Colonisation of soil is not only affected by the soil characteristics (Glaser and Lamar, 1995; Lamar et al., 1987) but also by the fungal growth substrate (also referred to as fungal carrier) and isolate (Leštan and Lamar, 1996). The pre-colonised growth substrate not only supports

the survival of white-rot fungi in the 'alien' soil-environment, but also needs to induce the enzyme activity required for biodegradation. Boyle (1995) concluded that additional growth substrate not pre-colonised by the white-rot isolate facilitates white-rot fungal growth in soils.

The aim of this project was to determine the effect of different parameters (soil type, soil moisture, pH, temperature, growth substrate concentration, fungal isolate and level of PCP contamination) on the colonisation of New Zealand soils by three native *Trametes versicolor* (Linnaeus) Pilat isolates.

Material and methods

Fungus and inoculum preparation

Three isolates of *Trametes versicolor*, HR131, HR154 and HR160 (HortResearch Culture Collection, Lincoln) capable of degrading PCP (Walter et al., 2003 – Chapter 2) were maintained as mycelial plugs in 7 ml bijou bottles (Samco Laboratories) of sterile distilled water at room temperature. Fungal inoculum was produced by transferring mycelial plugs from the bijou bottles onto potato dextrose agar (PDA, Difco) plates and incubating at 30°C for 6 days in the dark.

Soils and fungal growth substrate

Two arable soils were collected to a depth of 300 mm from the Canterbury region, New Zealand. The soils were a Temuka deep clay loam (Typic gley or Humaquept, according to the New Zealand soil classification or USDA taxonomy, respectively) and a Wakanui silt loam (Mottled immature pallic or Aquic Haplustepts, according to the New Zealand soil classification or USDA taxonomy, respectively). Soils were air dried and stored in plastic bags at 4°C.

Samples of PCP contaminated soil (non-sterile, approximately 30 years old from a former timber treatment facility) with average PCP residue levels of 5000 and 265 mg/kg, respectively, was sieved (< 1 mm) and diluted with sterilised Temuka deep clay loam to give PCP concentrations of approximately 0, 20, 50, 100 and 200 mg/kg (Walter et al., 2004 – Chapter 3).

Air dried fungal growth substrate (SCS) consisting of a *Pinus radiata* (D. Don) sawdust:corn meal:starch (75:8.3:16.7, C:N ratio = 130:1) mixture (Leštan and Lamar, 1996) was mixed with each soil at concentrations of 0, 25, 50, 75 and/or 100% (v/v).

The soil-growth substrate-mix (henceforward referred to as soil-mix) was sterilised by autoclaving twice (1 h, 120°C, 110 kPa) on two successive days.

The water holding capacity of each soil-mix was determined (McLaren and Cameron, 1996) for the corresponding container size used in the experiments and the amount of water required (per gram air dry soil-mix) calculated to reach 100, 80, 60 and 40% field capacity (–10 kPa).

pH effect

The effect of pH on fungal growth was studied in two pH experiments. In the first experiment the pH of the PDA was adjusted at 60°C with sulphuric acid and sodium hydroxide to reach final pH values of 2; 2.2; 2.4; 2.6; 2.8; 3; 4; 5; 6; 7; 8; 8.2; 8.4; 8.6; 8.8; 9; 9.2; 9.4; 9.6; 9.8 and 10. The agar was poured into sterile petri dishes (15 mL/dish). The cooled agar then was centrally inoculated with mycelial plugs (6 mm diameter), the mycelium facing the agar surface. Plates were set up in triplicates for each fungal isolate and incubated at 30°C in the dark for 7 days. Fungal growth was monitored by measuring mycelial extension in two perpendicular directions after 3 and 7 days of incubation.

In the second pH experiment, the pH of the two soils was modified as described by Bolan, et al. (1996) over a 4-week period. The soils, reaching pH values from 2-9 at a 60% field capacity, were then measured into sterile petri dishes (20 mL/plate) and each plate (triplicates) centrally inoculated with a mycelial plug (10 mm diameter) from isolate HR131, the mycelial side facing the soil. Plates were sealed with parafilm and incubated for 3 weeks at 30°C. Colonisation was measured as described in the section "Basic assay" after 7, 14 and 21 days incubation.

Basic assay

The soil-mix (100 mL) was measured into 300 mL tissue culture pottles (Propak), weighed, and soil-mix moisture adjusted to 40, 60, 80 and/or 100% field capacity by pipetting sterile tap water onto the surface. Pottles were sealed and left at 4°C for 16 h to allow the soil-mix to reach an even moisture equilibrium prior to inoculation with the fungi. The soil-mix was centrally inoculated with a mycelial plug (10 mm diameter), the mycelium facing the substrate. Inoculated pottles were re-sealed and incubated in the

dark at 30°C (unless specified) for 3 weeks. Treatments were set up in duplicate or triplicate blocks, with treatments being completely randomised within a block.

Fungal colonisation was assessed at weekly intervals (7, 14, 21 days) by measuring the growth area (1, 5, 10, 20 ...100% coverage, with 1% = no growth beyond mycelial plug) and visually estimating growth density using a scale of 1 (sparse), 3 (moderate) and 5 (dense), with moderate and dense being approximately 3 and 5 times, respectively, the density of sparse. Growth and viability of the fungal inoculum was checked by centrally inculating two PDA plates for each isolate and simultaneously incubating for one week at the temperatures used in the soil experiments.

Soil-mix experiments

Three soil-mix experiments were conducted sequentially. In Experiment 1a and 1b the effect of soil type (Temuka deep clay, Wakanui silt loam), fungal isolate (HR131, HR154, HR160), growth substrate concentration (0, 25, 50, 75, 100% SCS) and moisture level (40, 60, 80, 100% container capacity) was determined. In Experiment 1a non-autoclaved soil (but autoclaved SCS) was used, whereas in Experiment 1b the soil-mix was sterilised as described above. In both Experiments 1a and 1b the treatments were set up in triplicates as a fully factorial design with 4 factors (2 soils x 3 isolates x 5 SCS concentrations x 4 moisture levels).

Experiment 2 was a modified repeat experiment of Experiment 1b. The effect of soil type, fungal isolate, growth substrate concentration (25, 50%), moisture level and temperature (10, 20, 30, 40°C) on fungal soil colonisation was studied. The treatments were set up in duplicates as a fully factorial design with 5 factors (2 soils x 3 isolates x 2 SCS concentrations x 4 moisture levels x 4 temperatures).

Experiments were inoculated, incubated and assessed as described in the section "Basic assay".

PCP experiment

In the first PCP experiment, PCP-soils (non-sterile) were augmented with 25% sterile SCS (henceforward referred to as PCP-soil-mix), measured into the tissue culture pottles, moisture adjusted to 80% field capacity and then further inoculated with agar plugs from the white-rot fungi. The pottles were incubated and assessed as described in the basic assay. In a repeat experiment of this greater volumes of soil were used in

larger containers. The PCP-soil-mixes were measured (200 mL) into 2 L surface sterilised ice cream containers (170 x 170 x 80 mm^3). As before, the moisture of the PCP-soil-mix was adjusted to 80% field capacity. Each PCP-soil-mix was centrally inoculated with a completely fungal colonised SCS-plate, sealed with a fitting lid and incubated (positioned at random in the incubator) at 30°C in the dark for 4 weeks. The SCS-plate was produced by measuring 40 mL sterile SCS into a petri dish (60% field capacity), inoculating with 6 mm mycelial agar plug and incubating for 2 weeks at 30°C in the dark. Fungal colonisation of the PCP-soil-mix was measured as described above after 7, 14, 21 and 28 days incubation. Treatments were set up in duplicate for all three *T. versicolor* isolates.

Statistical analysis

For the soil experiments, correlation analyses were conducted between assessment types (area covered and mycelial density) and assessment times (7, 14 and 21 days). Analysis of Variance (ANOVA) was used to determine the main effects and interactions for the factors using Minitab version 12.1.

Results

pH experiments

The pH of the agar affected ($P<0.001$) the growth of the fungi, with no difference ($P>0.05$) between the three isolates studied (Figure 5.1) in the pH range of 4-7.

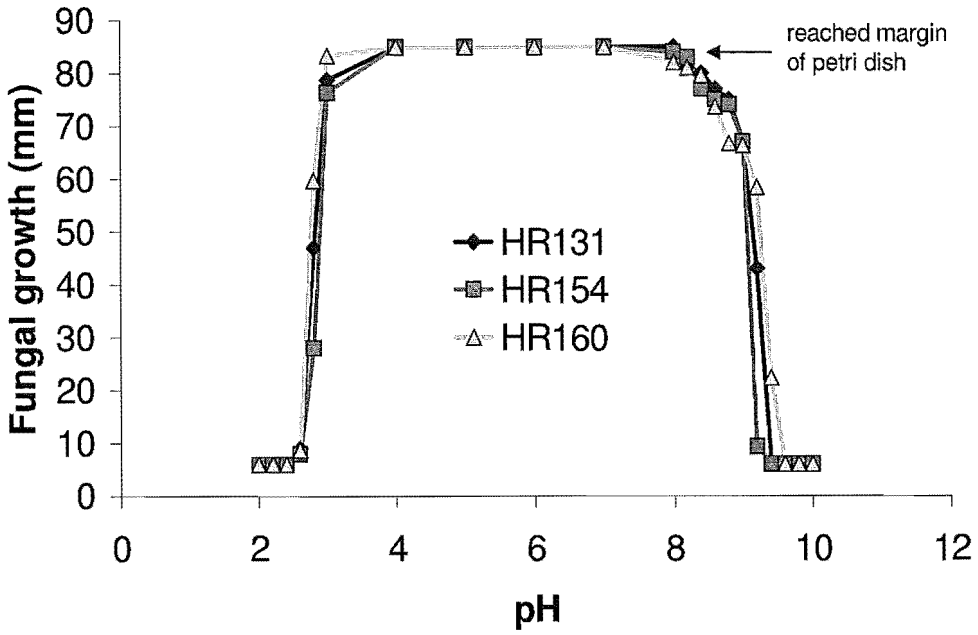


Figure 5.1. Effect of pH on the growth (diameter) of three *T. versicolor* isolates on agar after 7 days incubation at 30°C in the dark.

However, the fungal isolates showed different ($P<0.001$) tolerance levels (as expressed in mycelial growth) to the more acid or more basal agar media. After 7 days incubation, no fungal growth was observed at a pH of 2.4 or less and a pH of 9.6 or greater.

Both, pH and the soil type affected ($P<0.001$) the fungal colonisation of the two soils, with the Temuka deep clay showing two to three times the amount of fungal growth compared to the Wakanui silt loam. Mycelial density was sparse for all treatments. Mycelial extension was greatest for both soils at a pH range of 3.5-4.5, with no white-rot fungal growth (in both soils) at a pH lower than 3 or greater than 8.5.

Soil-mix experiments

In the two soil-mix experiments there was a very high correlation ($P<0.001$) between 'area covered' and 'mycelial density', the two assessments of fungal growth. Therefore the fungal colonisation results are presented predominantly for 'area covered'.

During incubation, fungal colonisation of soil-mixes increased ($P<0.001$) for all three fungi, with the fungal growth assessments correlating ($P<0.001$) between the three assessment times. Therefore data are presented for the 7 day assessment only, because

in some treatments, pottles were fully colonised by the white-rot fungi within 2 weeks of incubation. Further, after 3 weeks incubation some contamination with other organisms, such as *Trichoderma* spp., was observed.

The inoculum of the three white-rot fungi was viable in all experiments as shown by the growth of the isolates on PDA plates at all temperatures except at 40°C.

Soil-mix Experiment 1

In Experiment 1a, indigenous microbial populations, particularly soil-borne fungi such as *Fusarium* spp., *Trichoderma* spp., *Penicillium* spp. and *Rhizopus* spp., rapidly colonised (and sporulated) in the soil augmented with SCS. This did prevent visual assessment of colonisation by white-rot fungi, although visual assessments could be made in the soil only treatments. Thus the experiment was repeated using sterilised soil to facilitate the study on the effect of SCS concentration on soil colonisation by New Zealand native *T. versicolor* isolates.

In Experiment 1b, soil type ($F=134.5$; $P<0.001$), growth substrate concentration ($F=23.2$; $P<0.001$), soil moisture level ($F=12.2$; $P<0.001$), fungal isolate ($F=5.1$; $P<0.01$), influenced fungal colonisation of the soil-mix measured in 'area covered'. Similar results were obtained for the 'mycelial density' assessment. There were also significant interactions between soil type and isolate ($F=8.4$; $P<0.001$), and soil type and soil moisture ($F=5.2$; $P<0.01$). The main effects are clearly shown in Figure 5.2. It is noteworthy, that white-rot fungi more readily colonised the Wakanui silt loam than the Temuka deep clay. Also, the presence of the fungal growth substrate was more important than the actual concentration of SCS. Equally, mean mycelial density was sparse (1.9) for 0% SCS compared to medium density (3-3.3) for augmented SCS at concentrations of 25-100%. Based on these results, the experiment was repeated (soil-mix Experiment 2), excluding 0% SCS (due to low fungal growth) and the two highest SCS concentrations, but including temperature as a new factor.

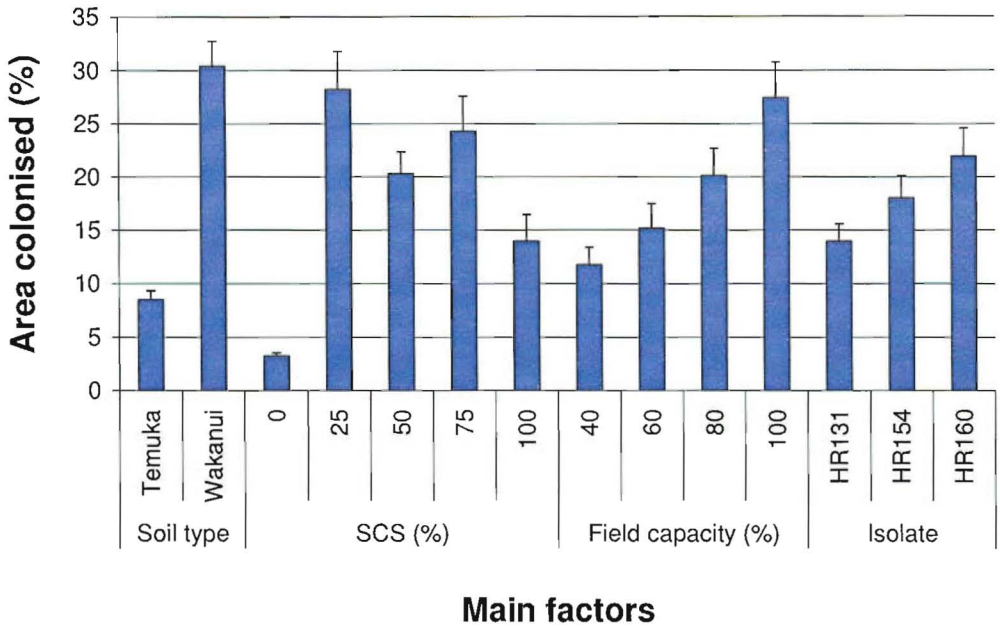


Figure 5.2. Influence of soil type (Temuka deep clay loam, Wakanui silt loam), fungal growth substrate (SCS) concentration, water content (% field capacity) and *T. versicolor* isolate on white-rot fungal soil-mix colonisation incubated for 7 days at 30°C in the dark. Error bars indicate standard deviation of the means. Results are from Experiment 1b.

Soil-mix Experiment 2

At 10°C, after 7 days incubation, the maximum fungal coverage was 5%, with a maximum coverage of 30% after 21 days incubation for selected treatments. At 40°C, for 76% of all treatments, no fungal growth was observed, not even colonisation of the agar plug itself. The reduced growth at 10°C and almost complete lack of growth in the soil-mix at 40°C, was confirmed with the control-agar plates. Because of the limited growth from treatments at 10°C and 40°C, results are presented for ANOVA for 20°C and 30°C (Table 5.1).

Table 5.1. Main effects and interactions of fungal carrier (SCS) concentration, temperature, soil type, soil moisture and *T. versicolor* isolate on soil colonisation by New Zealand white-rot fungi as shown by ANOVA (General linear model, non-significant (F<5) interactions are not shown). Results are from Experiment 2

Factor	Degrees of freedom (df)	Mean Square	F-value	Significance P ≤
SCS concentration (SCS)	1	4482	36.2	0.001
Soil type	1	8564	70.2	0.001
Temperature (Temp.)	1	20756	170.0	0.001
Soil moisture (Moisture)	3	4482	36.7	0.001
Fungal isolate	2	172	1.4	0.246
SCS * Temp.	1	2865	23.5	0.001
Soil type * Temp.	1	3487	28.6	0.001
Moisture * Temp.	3	2147	17.6	0.001
Soil type * Moisture	3	1812	6.7	0.001
Soil type * Temp. * Moisture	6	625	5.8	0.001
Error	96	2379		
Total	192			

SCS concentration = 25; 50% (v/v)

Soil type = Temuka deep clay; Wakanui silt loam

Temperature = 20; 30°C

Soil moisture = 100; 80; 60; 40% field capacity of the soil-mix

Fungal isolate = *T. versicolor* isolates HR131; HR154; HR160

Analysis of variance showed, that all factors, except fungal isolate, significantly influenced soil colonisation, and significant interactions between the different parameters studied occurred (Table 5.1). The main effects are further illustrated in Figure 5.3.

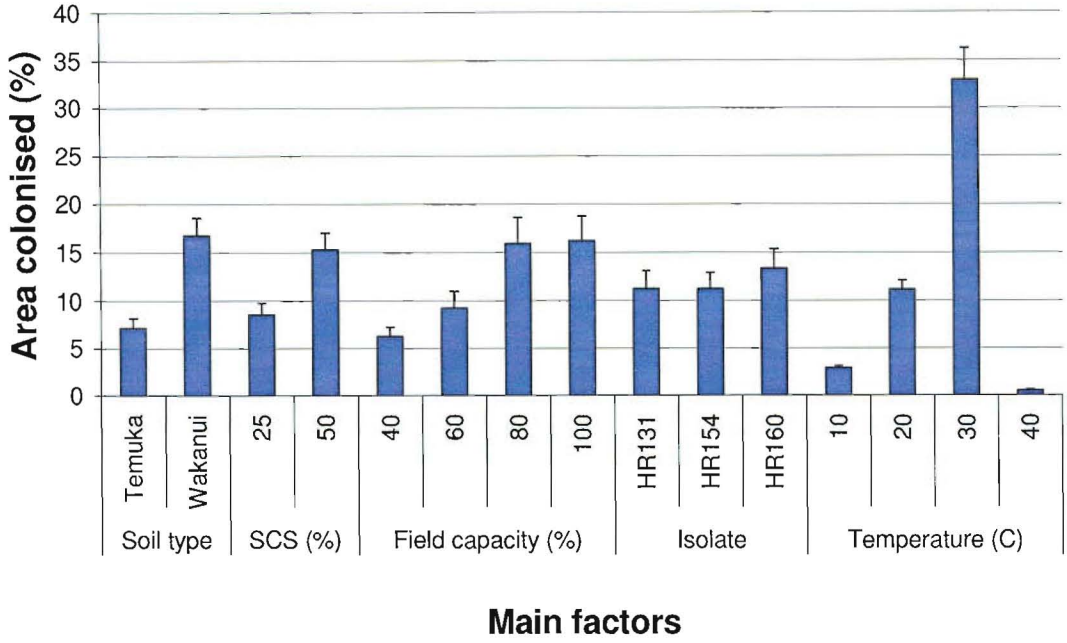


Figure 5.3. Influence of the soil type (Temuka deep clay loam, Wakanui silt loam), fungal growth substrate (SCS) concentration, water content (% field capacity), *T. versicolor* isolate and temperature (°C) on white-rot fungal soil-mix colonisation incubated for 7 days in the dark. Error bars indicate standard deviation of the means. Results are from Experiment 2.

PCP experiment

Inoculation of PCP-soil-mixes with an agar-plug of *T. versicolor* isolates did not facilitate fungal colonisation as observed in the earlier soil-mix experiments, particularly for PCP levels ≥ 50 mg/kg. Therefore experiments were repeated with a larger fungal inoculum biomass.

Colonisation of PCP contaminated soil by white-rot fungi increased ($P<0.01$) during incubation (Figure 5.4). There was a high correlation ($P<0.001$) between the four assessment times. PCP concentration ($F=75.9$; $P<0.001$) and isolate ($F=11.4$; $P<0.001$) affected colonisation as measured by 'area covered'. In contrast 'mycelial density' was not affected by PCP level ($F=1.9$; $P>0.05$) but was affected by isolate ($F=6.6$; $P<0.01$). The rapid decline in fungal colonisation in response to increasing PCP levels is clearly shown in Figure 5.4. There was no significant interaction between isolate and PCP concentration for fungal PCP-soil-mix colonisation ($F=0.85$, $P=0.538$).

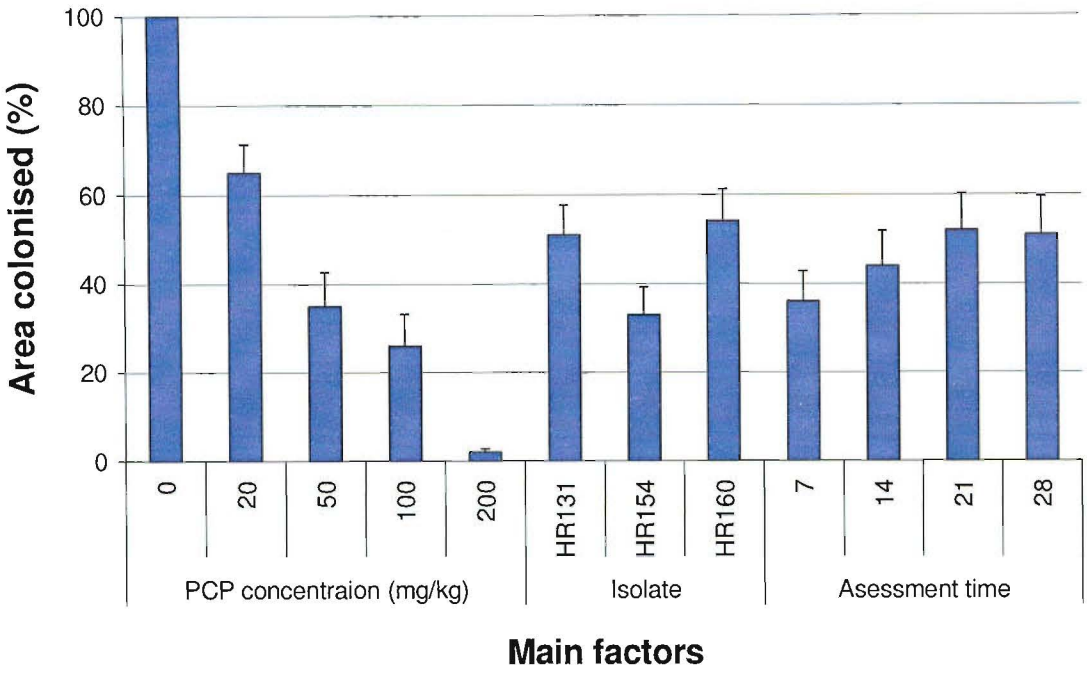


Figure 5.4. Influence of PCP concentration, *T. versicolor* isolate and assessment time (days) on white-rot fungal PCP-soil-mix colonisation incubated at 30°C in the dark for 28 days. Error bars indicate standard deviation of the means.

Discussion

Mycelial growth of three *T. versicolor* isolates studied was affected by pH, but all isolates showed a high tolerance for pH, with small (but significant) isolate differences at the lower or higher pH levels. White-rot fungi are known for their ligninolytic activity at low pH levels with a physiological pH of 4.5 for *Phanerochaete chrysosporium* (Tuisel et al., 1990). The *T. versicolor* isolates were able to readily grow on agar and colonise soils at a very wide pH range. This confirms the findings of Lamar et al. (1987) and Boyd-Wilson et. al (2005 – Chapter 6) who found no relationship between the growth of white-rot fungi and soil pH. Since most soils are within the pH range (McLaren and Cameron, 1996) where white-rot fungi grew in this study, pH adjustments may not be required in fungal bioremediation.

Temperature, as expected, influenced fungal soil colonisation by fungi. Most fungal inoculum was killed at 40°C in the soil-mix and all on agar. This confirms earlier findings by Walter et al. (2003 – Chapter 2) indicating that growth optimum for *T. versicolor* isolates is at 25-30°C. The *T. versicolor* isolates studied did not tolerate

higher temperatures, unlike the white-rot fungus *P. chrysosporium*, which has a temperature optimum of 39°C (Kirk et al., 1978). At 35°C, the three *T. versicolor* isolates still grew at approximately 80% of their growth rate observed at 30°C (unpublished data). This means that for bioremediation using *T. versicolor* biopile temperatures should remain below 40°C.

Temperature obviously determines fungal growth rate, but interestingly there were interactions between temperature and soil type, fungal growth substrate and soil moisture. This suggests that temperature optimum for *T. versicolor* colonisation is also influenced by these other factors. To determine the exact relationships and nature of these interactions more research should be conducted.

Next to temperature, soil type was the most important factor influencing fungal colonisation, as shown by the F-values. Lamar et al. (1987) also found that soil type affected soil colonisation by *P. chrysosporium*, as well as pollutant degradation. This effect of soil type occurred even though both arable soils were locally sourced and are very similar in their chemical soil properties when augmented with SCS (Boyd-Wilson et al., 2005 – Chapter 6). Therefore, Boyd-Wilson et al. (2005 – Chapter 6) further investigated the effect of soil properties on *T. versicolor* growth in 11 different New Zealand soils.

Increasing soil moisture improved fungal colonisation of the soil. This may be due to increased bioavailablity of nutrients by increasing soil moisture. Mycelium not only grew on the surface of the soil-mix, but also grew throughout the microcosms (data not presented) even at 100% field capacity. This indicates that soil aeration (at least at this microcosm level) was still adequate at 100% field capacity for white-rot fungi to colonise the soil mix. Aeration in biopiles is important because oxygen is required to support fungal growth as well as enzyme activity in bioremediation processes (Holroyd and Caunt, 1995). No research publications appear to discuss the role of soil moisture for soil colonisation by white-rot fungi. However, water potential is known to play an important role in wood decay by white-rot fungi (Griffin, 1977). Field capacity was a useful standard unit expressing soil water content, because the large amounts of fungal growth substrate interfered with standard soil moisture measurements (McLaren and Cameron, 1996). Suction plates were not used due to the large scale of the experiments. The amount of fungal growth substrate in the soil-mix was important, but more in terms

of its presence or absence, indicating that the amount of organic matter was not the rate-limiting factor for white-rot fungal growth.

The *T. versicolor* isolates colonised non-sterile soil without the presence of the growth substrate SCS (e.g. pH-experiment, soil-mix Experiment 1a, PCP experiment), however by adding the SCS, which contains starch and corn meal, indigenous fungi quickly colonised the soil-mix, except when PCP was present. Boyle (1995) suggested that additional growth substrate may be beneficial for soil colonisation by white-rot fungi. This, as mentioned, was true for sterilised soil and non-sterile soil containing PCP. No growth of indigenous fungi was observed in the PCP-soil-mixes, probably because of the fungicidal nature of the PCP. It is likely that the indigenous soil fungi did not survive the initial PCP contamination, nor were they able to re-colonise the polluted soil over the 30-40 year aging process. The research presented here showed that a small piece of white-rot fungal inoculum can colonise 100 mL of non-contaminated soil within 7 days if the conditions are suitable. Colonisation of contaminated soil however was slower and dependent on the PCP concentration. This is important when augmenting white-rot fungi to polluted soil, as the ability to colonise a particular substrate will determine the amount of fungal inoculum required and will therefore also affect treatment costs.

PCP concentration affected fungal colonisation. While the three *T. versicolor* isolates could grow on PCP amended agar (200 mg/kg, Walter et al., 2003 – Chapter 2), a similar PCP concentration nearly reduced all fungal growth. For one isolate (HR131) even the large SCS-plate inoculum completely died. However, isolate HR154 started to colonise the soil at this concentration after 28 days (data not presented). Alleman et al. (1993) reported that the amount of mycelial biomass influences the tolerance of white-rot fungi to PCP. This was also observed by Walter et al. (2003 – Chapter 2) and again confirmed in this study where the mycelial plug inoculum did not support fungal colonisation of the PCP-soil-mix, but the SCS-plate inoculum did.

While the fungal isolate affected soil colonisation, it was the least important factor and was only significant in one of the soil-mix experiments. There were no interactions between fungal isolate and the other factors studied, indicating that growth of the three *T. versicolor* isolates was affected similarly by the different soil parameters studied. In the PCP-experiment, isolate HR154 showed the least amount of PCP-soil-mix colonisation, but as mentioned above it was the most tolerant to the higher PCP levels.

All three isolates have been shown to degrade PCP *in vitro* (Walter et al., 2003; 2004 – Chapters 2, 3).

Colonisation of soil is an important first step in bioremediation using white-rot fungi, although colonisation does not equate to pollutant degradation. Colonisation determines the amount of fungal inoculum required, and hence affects the overall treatment costs. For successful bioremediation in the field good colonisation of the polluted soil is desirable and therefore it is necessary to identify the rate-limiting factors for colonisation and bioremediation. Temperature being the main driver for colonisation, can be controlled in biopiles. Equally soil moisture can be artificially managed (Wise et al., 2000). The amount of growth substrate required to maximise fungal colonisation for any particular isolate can be determined in laboratory based treatability tests and manipulated in the field. However the effect of soil type on colonisation and ultimately pollutant degradation is not fully understood and needs to be further investigated.

Acknowledgements

We thank the Foundation for Research, Science and Technology for funding and Dr Chris Frampton for statistical advice.

Additional information[A]

The effect of isolate and soil moisture are further illustrated in Figures 5.5 and 5.6, respectively.

[A] To the reader of this thesis, this section provides additional information that was not included in the scientific publication to be submitted, but of course is part of the PhD research itself and may aid in the understanding of the results.

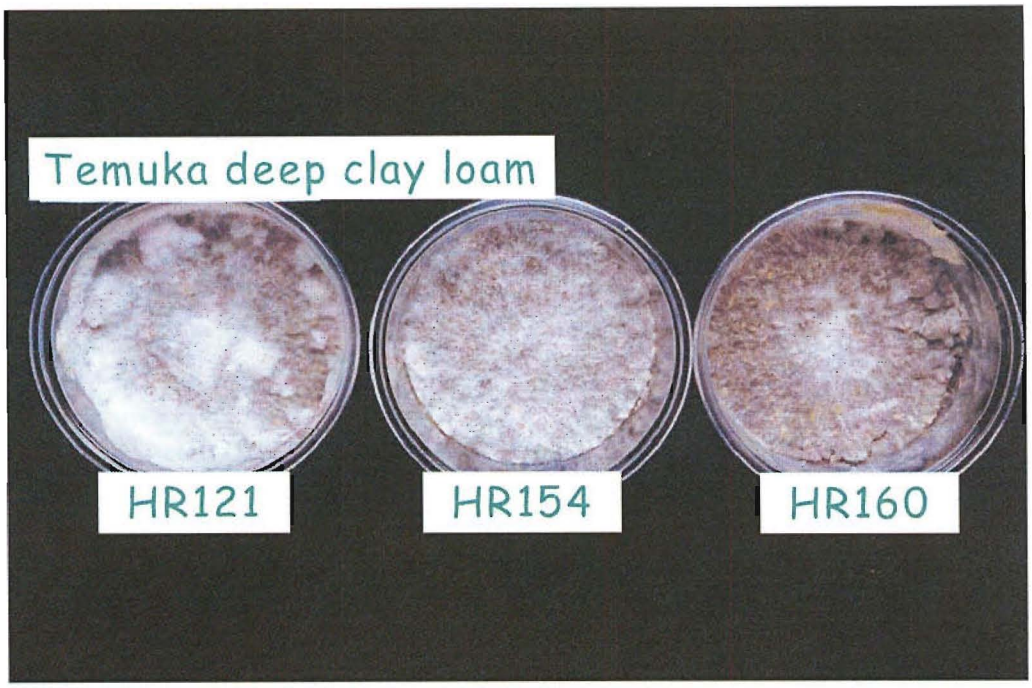


Figure 5.5. Fungal colonisation of soil (Temuka deep clay loam) by three different *T. versicolor* isolates (HR121, HR154, HR160) after 7 days incubation at 30°C in the dark. Results from Experiment 1b.

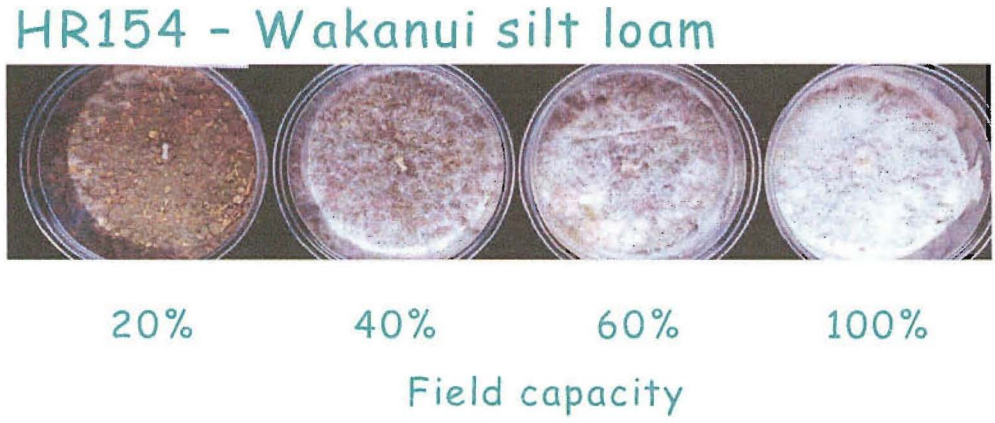
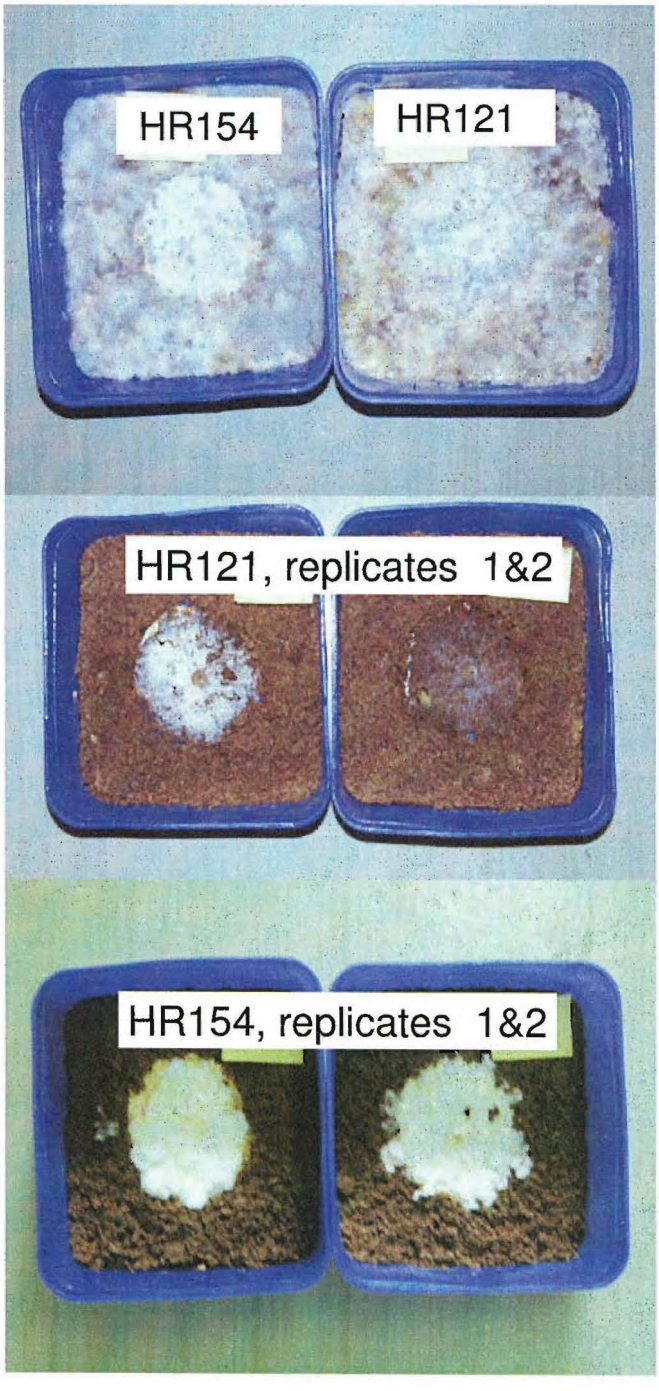


Figure 5.6. Fungal colonisation of soil (Wakanui silt loam) at different soil moisture levels (% field capacity) on by *T.versicolor* isolate HR154 after 7 days incubation at 30°C in the dark. Results from Experiment 2.

The steep decline in soil colonisation with increasing PCP concentration by *T. versicolor* isolates is further pictured in Figure 5.7., which clearly shows the

differences in fungal colonisation of uncontaminated and PCP-contaminated (200 mg/kg) soil.

Shivangini Chand, a student at University of Canterbury unde supervision of M. Walter, has further investigated the effect of moisture on colonisation of different substrates (soil, sand, sawdust) by *T. versicolor* HR121 using tension plates (and desorption) to create different moisture levels. The work by S. Chand, assessing fungal colonisation visually and enzymatically (biological potential and laccase), confirms the findings presented here.



Uncontaminated soil

PCP-contaminated soil

PCP-contaminated soil

Figure 5.7. Fungal colonisation of uncontaminated and PCP-contaminated soil (200 mg/kg) by *T. versicolor* isolates (HR121, HR154) after 28 days incubation at 30°C in the dark.

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Chapter 6

Growth of *Trametes versicolor* in New Zealand soils

Foreword

Chapter 6, like Chapter 5, studied soil factors that influence fungal colonisation by selected white-rot isolates. Chapter 5 focused on various soil factors (e.g. water content, pH, temperature), whereas Chapter 6 examined the effect of soil type and their corresponding soil properties on *Trametes versicolor* growth in soil. As mentioned in Chapter 5, Chapters 5 and 6 were conducted under the leadership of M. Walter, with technical assistance from K.S.H. Boyd-Wilson and J.H. Perry. The design of the experiments was done by M. Walter, with guidance from the Lincoln University biometrician C.M. Frampton, but the more complex statistical analyses were conducted by P. Alspach of HortResearch. The corresponding soil colonisation data is listed in Appendix to Chapter 6. A modified version of the chapter below, including additional research by by K.S.H. Boyd-Wilson will be submitted for publication. (journal still to be decided).

Abstract

Three isolates of the white-rot fungus *Trametes versicolor* were tested for growth on 11 soils collected from around New Zealand. The mycelial area covered and mycelial

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Modified publication by

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Modified version to be submitted in conjunction with subsequent work Kirsty Boyd-Wilson and Kathrin Schmidt (journal still to be decided).

density were assessed as a measure of growth. Growth was significantly dependent on soil type but not isolate. Principal component analysis and linear correlation studies between soil properties (after addition of a fungal growth substrate) and growth are reported.

Keywords: white-rot fungi, pentachlorophenol, bioremediation, biodegradation.

Introduction

The white-rot fungus *Trametes versicolor* (Linnaeus) Pilat is a saprophyte or secondary invader of woody tissue and is widely distributed in New Zealand native forests and urban areas (Horne, 2000). *T. versicolor* isolates have shown to degrade a range of environmental soil pollutants including the polycyclic aromatic hydrocarbons, anthracene and benzo[a]pyrene (Field et al., 1992; Collins et al., 1996), polychlorinated biphenyls (Zeddel et al., 1993; Beaudette et al., 2000) and pentachlorophenol (PCP) (Logan et al., 1994; Tuomela et al., 1999). Of six species of white-rot fungi examined, Alleman et al. (1992) concluded that *T. versicolor* was the best choice to degrade PCP in soils.

In New Zealand, PCP was used extensively by the forestry industry from the late 1940s to prevent sapstaining of wood. New Zealand was a heavy user of PCP because of the predominance of radiata pine which is a soft timber, and is more susceptible than most tree species to sapstain fungi. PCP was also used domestically for moss and algae control, by farmers for home treatment of timber, industrially to control slime in cooling waters in pulp and paper production, and in mushroom culture as a soil and timber steriliser (Stevenson, 1992). Although the use of PCP by the forestry industry voluntarily ceased in 1988 it was not deregistered and import prohibited until December 1991. An estimated 5000 tonnes of PCP was used at approximately 800 timber treatment sites throughout New Zealand over a period of approximately 40 years (Finnbogason and St Quintin, 1994). This widespread use of the chemical resulted in a variety of waste streams including contaminated soils from drippage and spills.

White-rot fungi do not occur naturally in soils, but are able to colonise contaminated soils if provided with a nutrient reserve and oxygen (Leštan and Lamar, 1996). The ability of the fungus to colonise soils is an important factor in isolate selection when treating contaminated soils. The growth of *T. versicolor* in New Zealand soils and the effect of soil properties on this growth have not been previously reported.

The growth of three isolates of *T. versicolor* able to degrade PCP (Walter et al., 2003 – Chapter 2) in 11 New Zealand soils uncontaminated with PCP was assessed. Relationships between soil properties, after the addition of a fungal growth substrate, and fungal growth were examined.

Materials and methods

Fungus and inoculum preparation

Three isolates of *Trametes versicolor*, HR131, HR154 and HR160 (HortResearch Culture Collection, Lincoln) capable of degrading PCP (Walter et al., 2003 – Chapter 2) were maintained as mycelial plugs in 7 ml bijou bottles (Samco Laboratories) of sterile distilled water at room temperature. Fungal inoculum was produced by transferring mycelial plugs from the bijou bottles onto potato dextrose agar (PDA, Difco) plates and incubating at 30°C for 6 days in the dark.

Soils

Due to difficulties in obtaining a range of contaminated soils with the sample pollutant level and profile, uncontaminated soils were used. Soils were collected to a depth of 30 cm from 11 locations around New Zealand (Table 6.1). Soils were chosen based on their geological make-up and age (Hewitt 1993) to give a range of acidities, organic matter and nutrient contents. Soils were air-dried and stored at 4°C.

Soil preparation and assessment

A fungal growth substrate (SCS) consisting of a *Pinus radiata* sawdust:corn meal:starch (75:8.3:16.7, C:N ratio = 130:1) mixture (Leštan and Lamar, 1996) adjusted to 50% gravimetric water content, was mixed with each soil at a ratio of SCS:soil = 1:4 by volume. A sample of each soil-SCS mixture was analysed for soil properties by RJ Hill Laboratories Limited, Hamilton (Table 6.2). The soil-SCS mixtures were sterilised by autoclaving at 121°C and 103.5 kPa for 1 h twice (on successive days), saturated and left to drain to reach container capacity. Tissue culture pottles (50 mm height x 100 mm diameter) were filled with 40 mL of soil-SCS and aseptically centre-inoculated with a

10 mm diameter plug of the white-rot isolates (mycelial side up). Pottles were sealed and incubated at 30°C in the dark.

Table 6.1. Description of soils used in this research

Soil type	**New Zealand soil classification**[a]	**USDA taxonomy**[b]
Bealey silt loam	Allophanic brown	Dystrudept
Cass soil – high country yellow brown earth	Acid brown	Dystrudept
Kaipaki peaty loam	Mesic organic	Medihemist
Hamilton clay loam	Orthic granular	Haplohumult
Horotiu sandy loam	Orthic allophanic	Hapludand
Pakaroa hill soil	Acidic orthic brown	Dystrudept
Takahe silt loam	Fragic pallic	Haplustept
Tamahere soil	Artifact fill anthropic	Haplanthrept
Temuka deep clay loam	Typic gley	Humaquept
Unnamed	Fluvial raw	Ustifluvent
Wakanui silt loam	Mottled immature pallic	Aquic Haplustepts

[a] Hewitt (1993)

[b] Soil Survey Staff (1998)

After 7 days incubation, the area (%) of the visible soil-SCS mixture covered by white-rot mycelium was visually estimated and the density of mycelial growth was scored using a rating system (1 – scattered hyphal growth; 3 – medium coverage of soil by hyphae (three times the density of 1); 5 – dense coverage of soil by hyphae (five times the density of 1)).

Table 6.2. Soil properties of each soil-SCS mixture

Soil series	pH	CEC (cmol/kg)	BS (%)	OM (%)	N (µg/g)	Ca	Mg	K	Olsen P (µg/ml)	S (µg/g)
						(cmol/kg)				
Bealey	4.8	16.1	9	14.2	6	0.7	0.40	0.34	7	30
Cass	5.4	15.8	23	16.3	6	2.3	0.67	0.57	18	6
Kaipaki	5.4	13.4	41	16.7	6	3.6	1.18	0.55	8	7
Hamilton	6.0	14.0	63	12.2	71	3.7	2.80	2.03	6	26
Horotiu	6.1	13.1	63	17.3	93	4.7	1.8	1.67	9	38
Pakaroa	6.1	14.2	64	13.9	33	5.1	2.76	1.05	10	38
Takahe	5.5	11.6	55	8.0	6	3.1	2.42	0.46	5	5
Tamahere	5.4	18.0	42	9.9	9	1.7	0.61	0.87	10	8
Temuka	5.7	16.5	82	11.8	6	9.6	2.83	0.82	35	30
Unnamed	5.8	2.2	100	3.1	6	1.6	0.34	0.23	6	2
Wakanui	5.8	11.2	63	10.8	6	5.3	0.61	0.99	45	5

CEC= cation exchange capacity; BS = base saturation; OM = organic matter

Experimental design and analysis

Due to the scale of the experiments, it was not possible to test all soils in the same experiment. Thus, five soils were tested in one group of experiments and the remaining six soils in another. Each group consisted of two sequential experiments with two complete replicates arranged in a randomised complete block design. The two groups of experiments were analysed separately as randomised complete blocks with four replicates: two for each of the two experiments. These separate analyses indicated some significant effects of experiment which rendered it unjustifiable to combine the two groups. Variates analysed were mycelial cover and density. Residuals from the analyses were examined to check for normality, variance homogeneity and outliers.

Principal component analysis was conducted on the correlation matrix of the soil characteristics. The scores of the first few principal components (PC) and the individual characteristics were plotted against the two white-rot growth variates for each isolate separately. Linear correlations were computed and tested for differences. Data was analysed using the S-Plus statistical package.

Results

Analyses of variance

Residual plots were generally satisfactory, with only one analysis giving any cause for concern. This was due to the presence of one extreme value. Exclusion of this value resulted in satisfactory residual plots, but made only minor changes to the means, standard errors and significance levels. Thus, results from the complete dataset are presented herein. Table 6.3 shows the mean mycelial cover and density results for the three *T. versicolor* isolates and 11 soil types for the two soil groups studied.

Table 6.3. Mean mycelial cover and density for three *T. versicolor* isolates and for 11 soil-SCS mixtures after 7 days for the two soil groups

	Mycelial cover (%)		**Mycelial density**	
	Group I	**Group II**	**Group I**	**Group II**
Isolate				
HR131	48.0	65.4	2.30	3.08
HR154	47.5	67.9	2.60	3.42
HR160	67.0	76.7	2.70	3.08
LSD (5%)	13.7	11.6	0.60	0.65
Soil series in mixture				
Kaipaki	47.5		2.67	
Hamilton	54.2		2.33	
Horotiu	34.2		2.17	
Pakaroa	75.8		2.67	
Tamahere	59.2		2.83	
Bealey		14.2		2.33
Cass		55.8		1.83
Takahe		69.2		3.17
Temuka		92.5		4.33
Unnamed		88.3		3.33
Wakanui		100.0		4.17
LSD (5%)	17.7	16.3	0.78	0.93

In many cases the experiment/replicate effect on white-rot growth was statistically significant. Examination of the means revealed this to be due to the difference between experiments rather than replicates within an experiment. Therefore combining the two groups of experiments was deemed unjustifiable. As a result, direct comparisons between the growth of white-rot on the two groups of soils cannot be made.
For both groups of soil, mycelial cover was different ($P<0.05$), but density was not different ($P>0.05$) between isolates (Table 6.3). There were significant differences between soils in mycelial cover for both soil groups and mycelial density for group II (Table 6.3). For all three isolates, mycelial cover correlated ($P<0.05$) with mycelial density. There were no significant interactions. The effect of soil type on fungal colonisation of the soils is clearly demonstrated in Figure 6.1.

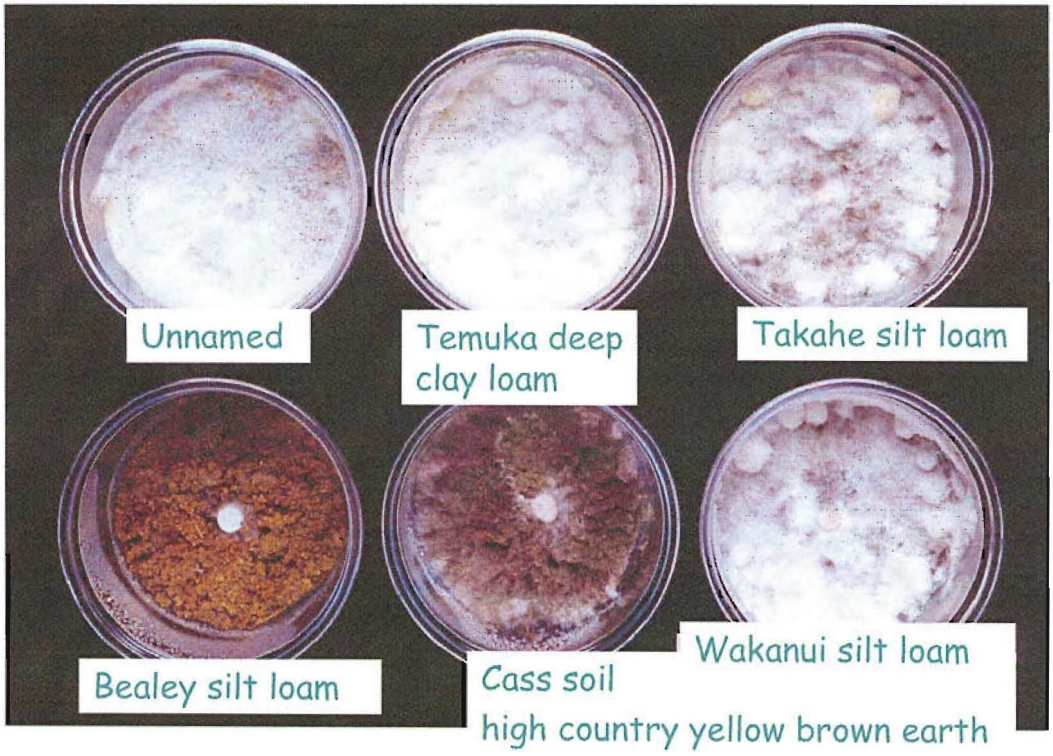

Figure 6.1. Fungal colonisation of different soil types (100% field capacity) by *T.versicolor* isolate HR121 after 7 days incubation at 30°C in the dark.

Relationships of white-rot growth with soil properties

The first principal component (PC) had positive loadings for all variates and could thus be regarded as some measure of overall soil fertility. This did not correlated with mean mycelial cover (r=0.029) and mean mycelial density (r=-0.029). Mean mycelial cover was positively correlated with the second PC (r=0.782). However, this PC did not have an obvious interpretation in terms of soil chemistry, being a contrast between [pH, percentage base saturation, calcium] and [cation exchange capacity, organic matter content and sulphur].

Although significant correlations occurred between fungal growth and certain soil properties for 1 of the 3 isolates, the only correlation that occurred for all 3 isolates was between mycelial cover and percentage base saturation (Table 6.4). There was a negative relationship between fungal growth and organic matter content.

Table 6.4. Correlation coefficients between selected soil properties and mycelial cover and density

	HR131		**HR154**		**HR160**	
	Cover (%)	**Density**	**Cover (%)**	**Density**	**Cover (%)**	**Density**
Base saturation (%)	0.673*	0.577	0.657*	0.365	0.810**	0.633
Organic matter (%)	-0.661*	-0.684*	-0.505	-0.160	-0.523	-0.595
Calcium (cmol/kg)	0.376	0.323	0.575	0.667*	0.593	0.564
Phosphorous (µg/ml)	0.512	0.504	0.682*	0.848***	0.580	0.404

***, **, * significant at P<0.001, 0.01 and 0.05

Discussion

In this study, growth could not be used as a criterion to select suitable isolates since all three isolates grew equally in the 11 soils. A better criterion for isolate selection may be growth in contaminated soil, however, due to the reasons mentioned earlier, only uncontaminated soils were used.

Because white-rot fungi do not grow well in unamended soil, particularly if it is not sterilised (Boyle 1995), the soils were amended with organic matter and nutrients in the form of sawdust, starch and cornmeal (SCS). There was considerable variation in mycelial cover and to a lesser degree, mycelial density across soils. Mycelial cover was correlated with mycelial density indicating that growth was even (in terms of these characteristics) in the soil. As PCP contaminated sites are found throughout New Zealand over a range of soil types, these results emphasise the importance for certain soil types of nutrient supplementation to ensure good fungal growth. Further research in this area is in progress.

The reason for the correlation between the percentage base saturation and mycelial cover is unclear. As percentage base saturation measures the proportion of the cation exchange capacity occupied by the cations Ca, Mg, K and Na, relationships between the CEC, the pH of the soil-SCS mixture and the fungal growth would have been expected. This was not the case. This confirms the findings of Lamar et al. (1987) who found no relationship between the growth of *Phanerochaete chyrsosporium* Burds. and the pH of three soils. The negative correlation between organic matter content and the fungal growth is surprising but could be accounted for by the lesser need of the fungus to grow through soils with a higher organic matter content.

It should be noted that sterilisation of the soils in this study removed the competition from other soil microorganisms, resulting in a higher nutrient supply than in a non-sterile soil (Cooke and Rayner, 1984). Addition of the SCS growth substrate would have further supplemented the soil's natural supply of nutrients. It is also possible that autoclaving the soils affected the soil properties (Skipper and Westermann, 1973). Analysis of the SCS-soil after autoclaving would have been useful. The effect of competition from soil microorganisms and growth of *T. versicolor* in unsterilised soil requires further investigation.

Acknowledgements

We thank the Foundation for Research, Science and Technology for funding, Frank Parry for collecting soils, and Dr Glyn Francis (Crop & Food Research), Dr Trevor Webb and Dr Malcolm McLeod (Landcare Research) for advice in selecting the 11 soils and for providing soils information.

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Chapter 7

Optimising remediation of PCP contaminated soil using white-rot fungi

Foreword

Based on the knowledge and information of the previous chapters (Chapters 2-6), in Chapter 7 the best performing New Zealand native white rot isolates were further studied to enhance bioremediation activity. The experiments conducted in this chapter also led to standardise the laboratory treatability tests. Development of treatability studies that are scientifically sound and commercially applicable was an important aspect of the student project. The work presented was led and conducted by M. Walter with technical assistance from K.S.H. Boyd-Wilson. The size of the experiments required technical assistance in order to set up a single treatability study within 2-3 days. All PCP residue analysis was contracted out to D. McNaughton (TELARC accredited analytical laboratory). Protocols for residue analysis are presented in Appendix Chemical Residue Analysis. ANOVA tables for the different experiments and PCP residue levels are presented in Appendix to Chapter 7. A modified version of the chapter below, has been published by International Biodeterioration and Biodegradation.

Modified publication by

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Laboratory trials on the bioremediation of aged pentachlorophenol residues. International Biodeterioration and Biodegradation (2005) Volume 55, Page 121-130.

Abstract

The variable nature of contaminated soil requires reliable assessments so called treatability studies, to optimise the bioremediation process in the field. Loboratory studies were designed here to select fungal isolates, determine optimum fungal inoculum concentration, determine optimum treatable contaminant concentration and predict degradation over time. Based on these experiments, laboratory studies were standardised.

Keywords: Treatability studies, bioremediation, PCP contaminated soils, white-rot fungi, New Zealand

Introduction

Bench-scale and pilot scale treatability studies are commonly employed to obtain site-specific performance data for biotreatment. Biological processes are dynamic in nature and more prone to site-specific influences than other remedial technologies (Bourquin and Pedersen, 1995). Therefore treatability studies are conducted to assess the contaminant and media characteristics related to treatment effectiveness.

Best practice in soil bioremediation has been described by Bewley (1996). Briefly, it requires the implementation of a series of protocols and procedures to fulfil project objectives in a cost-effective manner and within the regulatory frameworks. The issues that best practice soil bioremediation guidelines address are site investigation, feasibility analysis, remedial design, bioremediation implementation, verification and post-remediation monitoring. Treatability studies form an integral part of the bioremediation feasibility assessment that includes the determination of physical, chemical and microbial factors.

The aim of this research was to develop guidelines for laboratory based studies in order to (1) determine the degradation potential of soil pollutants by selected New Zealand native white-rot isolates; (2) determine the optimum fungal inoculum concentration required in the bioremediation process; (3) determine the optimum carrier (or formulation) used for the fungal inoculum; (4) determine the maximum treatable contaminant concentration; (5) determine the requirement and concentration of co-

ammendments (such as additional growth substrates, nutrients, surfactants, etc) for enhanced bioremediation; and/or (6) predict pollutant degradation over time.

Material and methods

A series of experiments was carried out sequentially to standardise the methodology for the laboratory tests. Pentachlorophenol (PCP) was used as the model contaminant in all tests. Except in the preliminary experiment (Experiment 1), all experiments used PCP contaminated soil (> 30 years old) from former and/or existing timber mill sites in New Zealand. Unless otherwise stated, Experiment 1 also describes the basic methodology used in subsequent experiments. An overview of experiments, soils, PCP residue levels and isolates used is given in Table 7.1. Isolates were chosen based on PCP degradation potential (Walter et al., 2003 - Chapter 2, 2004 - Chapter 3).

Table 7.1. Overview of fungal isolates, soil source, PCP concentration and residue type used in the five experiments

	PCP	Soil	Residue	Isolate code (HR-number)[a]							
Exp	**(mg/kg)**	**source**	**type**	**112**	**122**	**131**	**154**	**160**	**577**	**PC**	**PS**
1	0 - 2786	Top soil	Spiked			✓		✓			✓
2[b]	60	Mill-WKT	Aged	✓	✓	✓	✓	✓	✓	✓	✓
3	6266 - 26114	Mill-WPM	Aged			✓	✓		✓		
4	3629	Mill-AV[c]	Aged			✓		✓	✓		
5	1830 - 3629	Mill-AV	Aged	✓	✓	✓		✓	✓		
5b	1830 - 3629	Mill-AV	Aged	✓				✓			

[a] HR112, HR122 = unidentified isolates
HR131, HR154, HR160 = *T. versicolor* isolates
HR577 = *T. hirsuta* isolate
PC = *P. chrysosporium* isolate
PS = *P. sordida* isolate

[b] A total of 22 New Zealand native white-rot isolates were used in this study

[c] The AV 'soil' was a soil-less sawdust-based contaminated substrate

Experiment 1 (preliminary experiment)

The aim of Experiment 1 was to determine the effect of PCP concentration using spiked soil on contaminant degradation by selected native white-rot fungal isolates in comparison to an overseas white-rot fungal isolate.

Fungi. Two New Zealand isolates of *Trametes versicolor* (L.: Fr.) Pilat (isolates HR131 and HR160[A]) and one American isolate of *Phanerochaete sordida* (Karst) Erikss. and Ryv. (ATCC 90628) were maintained on 2% malt extract agar (MEA, Oxoid) slopes at 4°C. Isolates were transferred onto MEA plates and incubated at 30°C for two weeks in the dark. Fungal inoculum consisted of a *Pinus radiata* sawdust:corn meal:starch (75:8.3:16.7) mixture (SCS; Walter et al., 2003 – Chapter 2) adjusted to 50% moisture content (Leštan et al., 1996). SCS was sterilised by autoclaving in 250 mL polycarbonate specimen containers (LabServ) at 121°C and 110 kPa for 1 h twice, on successive days. The SCS was inoculated with a 6 mm mycelial plug from the MEA plate and incubated for 3 weeks at 25°C in the dark.

PCP. Non-sterile, screened top-soil (Temuka deep clay loam; Walter et al., 2004 - Chapter 3) was spiked with PCP (Aldrich, 98% purity). PCP was dissolved in acetone (40 mg/mL) and 163 mL of this stock was added to 2.6 kg sieved (4 mm) soil (20% moisture; w/w). PCP and soil were mixed thoroughly using a tumbler (5 min, approximately 200 rpm) resulting in a concentration of 2786 mg/kg PCP. The PCP spiked soil then was used for further soil PCP-inoculation by soil dilution (3, 10 and 30 fold), mixing with the tumbler. Aliquots of PCP contaminated soil (80 g dry weight; 80 mL) at concentrations of 2786, 929, 279, 93 and 0 mg/kg were then measured into 250 mL specimen containers (LabServ) and left uncovered in a fumehood to allow the acetone to evaporate. After one week, moisture loss was adjusted gravimetrically (by misting tap-water onto the soil surface with a hand hold sprayer) and PCP-soils were inoculated with the fungi.

Soil inoculation. PCP-soils were inoculated with 20% (v/v), the equivalent to approximately 5% (w/w, dry weights), of the fungal colonised SCS (SCSf). PCP-soil

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[A] Deposited at Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia with Accession numbers NM02/27875 and NM02/27877 for HR131 (Culture A) and HR160 (Culture C), respectively

and SCSf were mixed thoroughly by shaking the specimen containers. Controls consisted of un-inoculated PCP-soil (0% SCS) and 20% *T. versicolor* colonised, autoclaved SCSf (isolate HR160). All treatments were prepared in duplicate. Specimen jars were completely randomised and incubated in the dark at room temperature (23±3°C). Moisture was adjusted gravimetrically at weekly intervals as described above.

Assessment. After 42 days incubation, the colonised PCP-soil was disrupted and mixed with a spatula. Two 5 g samples were taken from each treatment for PCP residue analysis as described by Walter et al. (2003; 2004 – Chapters 2; 3). Main treatment effects were determined using analysis of variance (ANOVA) and treatment differences using Fisher's LSD test.

Experiment 2

The aim of this experiment was to investigate the ability of New Zealand native white-rot isolates tolerant to PCP *in vitro* to degrade PCP in aged-residue soil in comparison to two American cultures. Twenty-two New Zealand native isolates tolerant to 200 mg/kg PCP in agar (Walter et al., 2003 - Chapter 2) were compared to the two American isolates *Phanerochaete chrysosporium* Burds (ATCC 24725) and *P. sordida*. SCSf was produced for the 24 isolates as described above.

PCP, inoculation and assessments. Contaminated soil (60 mg/kg PCP) obtained from a former mill site (referred to as WKT soil) was sieved (4 mm), measured (80 mL; 51 g dry weight) into 250 mL specimen containers, inoculated with SCSf (32%, v/v) and incubated (pottles placed at random) in the dark at room temperature. Controls consisted of 0% SCS and autoclaved SCSf. All treatments were set up in duplicate. Moisture was maintained gravimetrically at weekly intervals. Fungal colonisation was assessed visually by estimating the area of visible mycelium (0, 5, 10, 25, 50, 75, 90 or 100%) after 7 days incubation. PCP residue levels were determined at day 0 (control treatments only) and after 42 days of incubation. Main effects and isolate differences in colonisation and PCP degradation were determined using ANOVA and Fisher's LSD test.

Experiment 3

The aim of Experiment 3 was to investigate the effect of surfactant and SCSf concentration on high levels of PCP degradation. Contaminated soil with a reported (data supplied from sawmill) average PCP concentration of 3200 mg/kg (40% moisture) obtained from a mill site (referred to as WPM soil) was sieved (4 mm), measured (80 mL; 51 g dry weight) into 250 mL specimen containers, amended with 5 concentrations of emulsifiable soybean oil (0, 1, 2, 3 or 5%; v/w) and then inoculated with SCSf (0, 15, 30, 45 or 60%; v/v) for three selected NewZealand native isolates (HR131, *T. versicolor* HR154[B], *T. hirsuta* HR577). Control treatments were the soil alone and autoclaved SCSf (HR131) treatments. Treatments were set up as a fully factorial design in duplicates and incubated at random in the dark at room temperature. Moisture was maintained gravimetrically and fungal colonisation was assessed visually in weekly intervals. PCP residue levels were determined at day 0 (control and HR131 treatments only) and after 42 days of incubation. Main effects and isolate differences in colonisation and PCP degradation were determined using ANOVA and Fisher's LSD test.

Experiment 4

The aim of this experiment was to study the colonisation of PCP contaminated organic matter and PCP degradation by selected white-rot fungi. Experimental protocols of Experiment 3 were followed. The 'soil' in this experiment was contaminated organic matter (3629 mg/kg PCP; 35% moisture) made-up predominantly of sawdust (particle size <2 mm) obtained from a former mill site (referred to as AV soil). The 80 mL volume in the specimen jars corresponded to 24 g dry weight. The three selected New Zealand native isolates used were HR131, HR160 and HR577 in comparison to an autoclaved SCSf control (isolate HR160).

Experiment 5

In Experiment 5 the effect of pollutant concentration of aged PCP residues and incubation period on PCP degradation by native white-rot fungi was investigated. The

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[B] Deposited at Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia with Accession number NM02/27876 for HR154 (Culture B)

methods of Experiment 3 were modified. The contaminated organic matter (AV soil) was used. Also, smaller specimen jars (75 mL; LabServ) were used, which decreased the starting volume from 80 mL to 50 mL. Five New Zealand native isolates (unknown HR112, unknown HR122, HR131, HR160 and HR577) were studied for PCP degradation in comparison to the American isolate *P. sordida*. The AV contaminated substrate was diluted with non-sterile top-soil (Temuka deep clay loam) to 100, 75 and 50% AV concentration. No emulsified soybean was added. All treatments were set-up in 5 replicates. PCP residues were analysed after 0, 42, 84 and 164 days of incubation, by destructively harvesting 3-5 specimen jars. Estimates of initial PCP levels were obtained from the controls only (from 5 replicate specimen jars). White-rot growth was monitored on five pots per treatment (these five pots were used for residue analysis at the end of the experiment). The area under the plot of white-rot cover was calculated for each specimen jar as a measure of fungal growth expressed as a white-rot growth quotient. The correlation between the resulting growth index and PCP residues was determined.

Experiment 5b

In this nested experiment, the effect of number of replicates assessed and residue sampling on variation of PCP recovery was determined for two randomly chosen treatments after 126 days incubation in order to decrease the high variation and improve cost-efficiency. Residue analysis was conducted by comparing individual PCP results of five replicate specimen jars from Experiment 5 versus combining the substrate of the five jars, thoroughly homogenising the content with a spatula and by re-sieving (2 mm) and removing two sub-samples for extraction and HPLC analysis (referred to as composite sample). Individual specimen jars were homogenised and sub sampled for the 'individual' residue analysis. Subsequently the five corresponding replicates of a selected treatment were combined, homogenised and sub sampled for the 'composite' analysis. These two types of sampling for residue analysis were conducted for (1) PCP-soil augmented with top soil (50%) and SCSf isolate HR112 and (2) PCP-soil augmented only with SCSf isolate HR160.

Results and discussion

Experiment 1

All PCP levels declined in all treatments after 42 days incubation. PCP levels were affected ($P<0.05$) by the treatments, with the highest PCP levels observed in the soil control (Figure 7.1). Only at the lower PCP starting concentrations (279 and 93 mg/kg) were differences ($P<0.05$) between the fungal treatments and the autoclaved SCSf control observed (Figure 7.1).

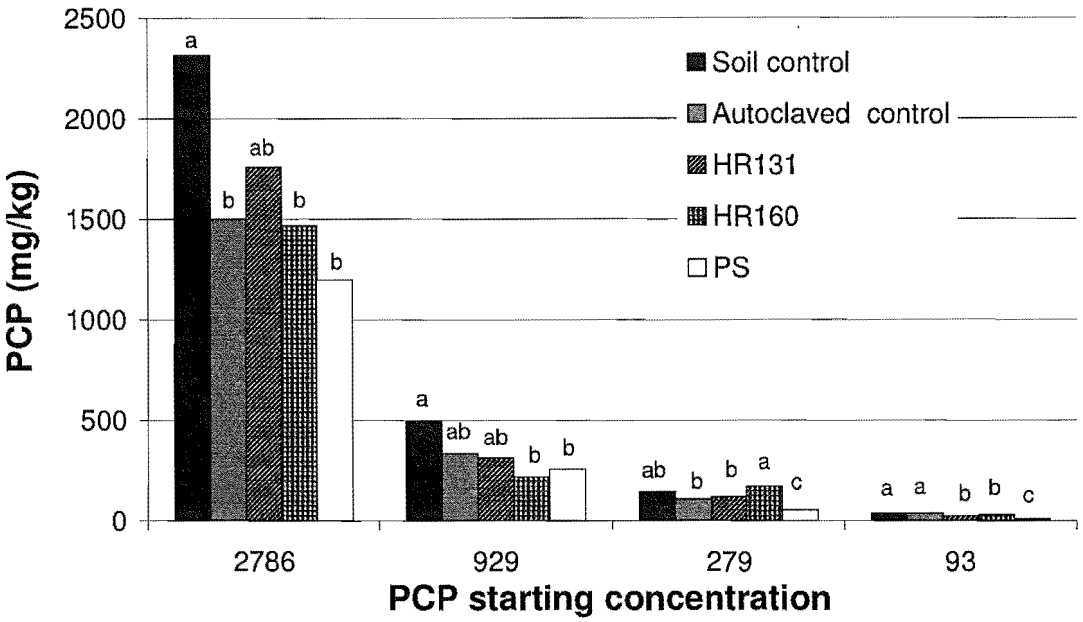

Figure 7.1. PCP decline in spiked soil (2786, 929, 279, 93 mg/kg starting concentrations) augmented with white-rot fungi after 42 days incubation at room temperature. Significant differences ($P<0.05$) between treatments are denoted by different letters (HR131, HR160 = *T. versicolor* isolates; PS = *P. sordida*).

Since PCP was added to non-sterile soil, the decline in PCP in the soil controls is not surprising. Some PCP may been lost in the volatile fraction during acetone evaporation or it may have adsorbed onto the equipment used. In addition, PCP may have adsorbed to the soil clays (Brixie and Boyd, 1994; Stapelton et al., 1994; Boyd et

al., 1988), soil organic matter (Shiu et al., 1994) or the organic matter introduced with the SCSf inocula and therefore may not have been extracted and measured. The augmented PCP may also have been decomposed by the endogenous soil microflora (McAllister et al., 1996). The level of decline suggests that biodegradation processes have occurred.

The almost complete lack of PCP degradation by the white-rot isolates tested compared to the autoclaved control is in disagreement to earlier findings (Walter et al., 2004 - Chapter 3) where up to 34% PCP mineralised in soil mesocosms. Overall, the American isolate *P. sordida* treatment showed slightly better PCP biodegradation potential than the New Zealand *T. versicolor* treatments. PCP was spiked, thus its bioavailability for microbial degradation will be greater than the bioavailability of aged residues (Alexander, 1999; Christodoulatous et al., 1994). PCP decomposition by soil microorganisms will be enhanced by providing a co-substrate (McAllister et al., 1996) such as supplied in the form of the autoclaved SCSf, consisting of killed fungal biomass, polysaccharides, celluloses, hemicelluloses and lignin. The role of white-rot fungi in the PCP decline observed was not elucidated. In earlier work Walter et al. (2004) found that PCP decline was not improved by the presence of white-rot fungi, but mineralisation rate was.

Without the use of tracers such as ^{14}C-PCP the fate of spiked PCP will be unclear, even if additional controls (such as sterilised soil, sterilised soil + autoclaved SCSf, and sterilised soil + SCS) are used, due to degradation, aging and partitioning processes. Additional controls or the use of tracers are not desirable methods for treatability studies in a commercial environment due to the costs involved (e.g. specialised equipment, additional residue analysis). Therefore it was decided to develop the methods for the laboratory studies using aged residues only. This also will be more representative of a commercial situation.

Experiment 2

Growth differed ($P<0.001$) between isolates and ranged from 17.5% colonisation to 100% colonisation of the visible SCS-soil mix. Nine of the isolates tested had degraded PCP significantly ($P<0.01$) after 42 days when compared to the control level at day 0. For isolate HR160, no PCP was detected in the sample after 42 days (Figure 7.2). Colonisation did not correlate ($P>0.05$) with PCP degradation. This is in agreement to earlier findings *in vitro* (Walter et al., 2003 - Chapter 2), where mycelial

growth of white-rot fungi did not correlate to PCP degradation. However, a high degree of degradation of PCP *in vitro* (up to 100%) was not predictive for aged PCP degradation in soil. For example, *in vitro*, isolate HR226 had no detectable PCP residues after 42 days stationary incubation (Walter et al., 2003 - Chapter 2), but this isolate did not show any significant PCP decline in the soil experiment. Selected New Zealand native isolates showed better degradation of aged PCP residues than the two American isolates *P. sordida* and *P. chrysosporium*. Neither American isolate showed significant decrease of PCP compared to the control treatment (autoclaved SCSf).

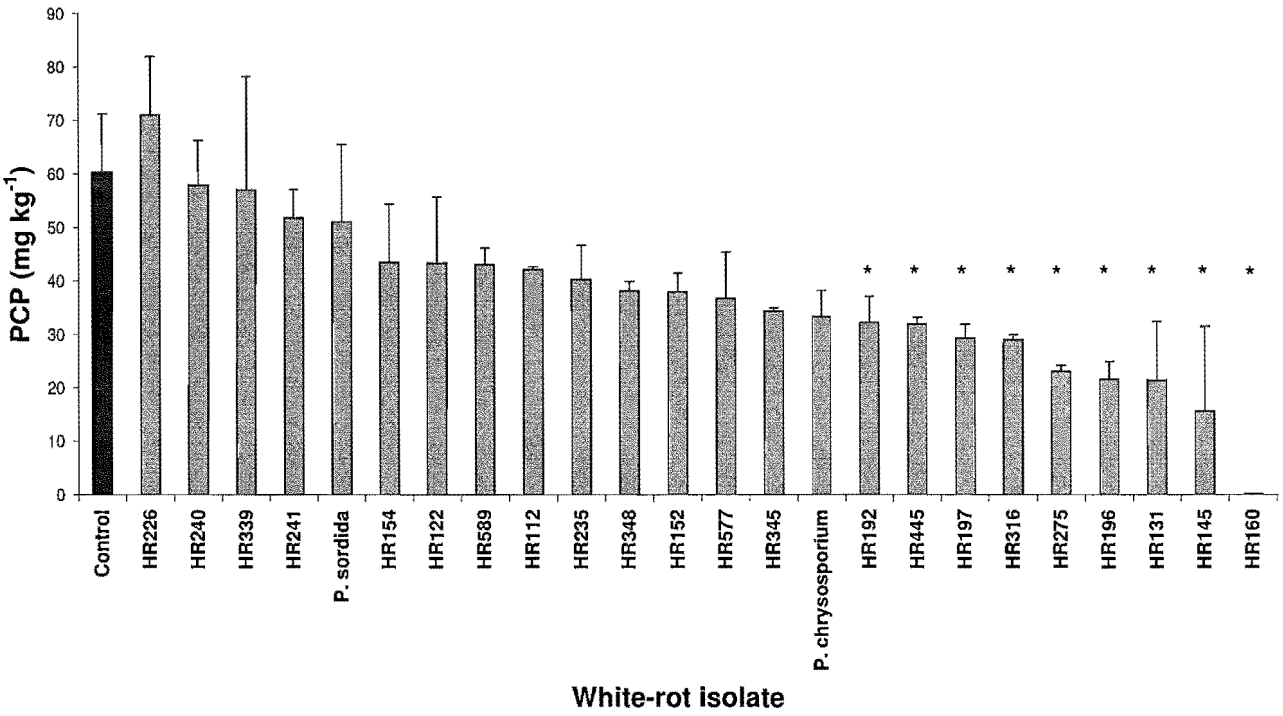

Figure 7.2. PCP remaining in soil after 42 days incubation with white-rot fungi at room temperature. Significant ($P<0.05$) differences from the control are denoted by *. Error bars indicate standard deviation of the means.

Soil factors and isolate play an important role in soil colonisation (Boyd-Wilson et al., 2005 - Chapter 6). While mycelial colonisation is not an indicator of PCP degradation, in field biopiles colonisation may be an important step to ensure PCP degradation throughout the biopile and may determine the amount of fungal inoculum required which would thus affect treatment costs.

Experiment 3

The reported PCP concentration of 3200 mg/kg for the WPM soil was found to be not reliable information. PCP analysis from Day 0 samples showed an average PCP concentration of 12696 mg/kg PCP (6266 – 26114 mg/kg PCP). This clearly indicates that residue information supplied by industry may not be reliable and the homogeneity of the soil-pollutant-SCSf mix needs to be vastly improved. A more even pollutant profile is required to standardise pollutant concentration in the replicate specimen containers. Based on this information, sieving and mixing practises have been intensified. Double sieving, and the use of a manual concrete mixer (Mitre 10) were employed to improve soil-pollutant homogeneity.

Irrespective of surfactant and SCSf concentrations, at these PCP residue levels (12696 mg/kg) virtually no fungal colonisation ($P>0.05$) and no PCP decline ($P>0.05$) could be observed during the 42 days incubation period. At these levels, the PCP residues in the WPM soil were most likely fungicidal. Even in specimen jars with the 'lower' PCP concentrations (6000-7000 mg/kg PCP), no fungal growth and no change in PCP levels could be observed over time.

The effect of surfactant on PCP extractability could not be determined due to the high variation of PCP levels in replicate specimen jars. No soil samples were collected directly before and after surfactant application.

Experiment 4

As expected, no white-rot fungal growth was observed in the autoclaved SCSf control treatment, thus that treatment was ignored in the further results description. At the level of PCP contamination studied, the highest soil colonisation observed was 10%.

PCP-soil colonisation by white rot fungi increased ($P<0.001$) over time, with the greatest fungal colonisation observed after 30 days. Soil colonisation was dependent on isolate ($P<0.001$), SCSf concentration ($P<0.001$) and the amount of emulsifieable soybean oil added ($P<0.001$). However, there was no isolate difference ($P>0.05$) in soil colonisation after 30 days incubation (Figure 7.3), explaining the interaction ($P<0.001$) between isolates and assessment times during the 30 day incubation period. No fungal growth occurred at an SCSf concentration of 15% (v/v), with no difference ($P>0.05$) in percentage soil colonisation between the other SCSf concentrations (30, 45 and 60% SCSf gave 5.7, 6.3 and 6.8% soil colonisation, respectively, after 30 days incubation). Similarly, the presence or absence of the soybean oil influenced fungal colonisation. For

example, fungal colonisation (pooled across all isolates and averaged over the 4 assessment days) at 0% soybean oil was half (2.1%) the colonisation observed for the other four soybean oil concentrations (4.1, 4.1, 3.9 and 4.7% colonisation for 1, 2, 3 and 5% soybean oil, respectively).

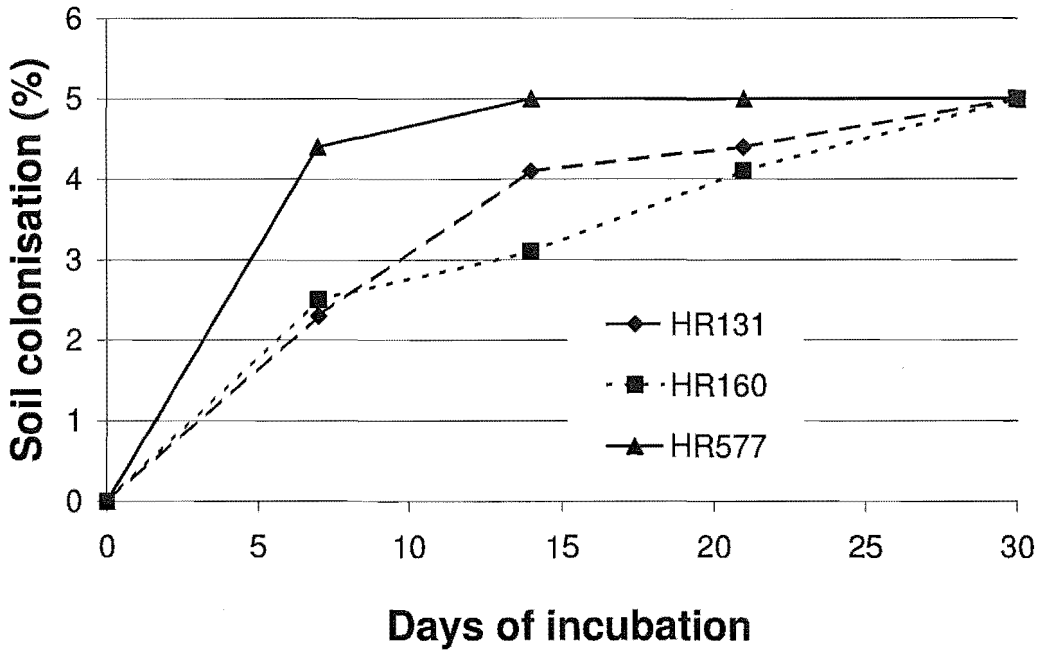

Figure 7.3: Average PCP-soil colonisation (%) by white-rot fungi. Values are averaged across all soybean oil concentrations for 30% (v/v) SCSf.

The work clearly shows that fungal colonisation of polluted soil is isolate dependent. Fungal inoculum concentration also needs to reach a threshold to facilitate the colonisation of soil by white-rot fungi. Surfactants/nutrient amendments such as the soybean oil also support white-rot fungal growth in contaminated soil. Overall, however, at the PCP level of 3629 mg/kg, comparatively little white-rot colonisation of the AV soil occurred. Toxicity of PCP to the white-rot fungi is a likely reason. In contrast, in Experiment 1, the high level of spiked PCP (2786 mg/kg) was not fungicidal to the white-rot isolates tested. Mycelial growth was observed in Experiment 1, but little to none mycelial growth was observed in Experiment 4. For aged residues, high level of PCP contamination (3629 mg/kg) proofed to be fungicidal. In contrast, for

spiked PCP, high levels of PCP contamination (2786 mg/kg) did not kill the fungal inoculum. This suggests, that it is important to carefully define white-rot fungal treatment PCP-thresholds using aged PCP residues.

The impact of PCP concentration on fungal colonisation and PCP degradation needed to be further investigated. Therefore, in Experiment 5, both soil colonisation and PCP degradation for different PCP concentrations was further examined.

Experiment 5

The fungal inoculated AV soil had water contents of 37, 47 and 57% for the 50, 75 and 100% substrate mix respectively. Over 164 days incubation, PCP levels declined ($P<0.001$) for all treatments at all AV soil concentrations. At the last assessment, the average PCP residue levels in 100, 75 and 50% AV soil ranged from 2987–4140, 689–1580, and 85–829 mg/kg PCP, respectively, for the different treatments. Only for 50% AV soil, were significant treatment differences detected due to the very large variation in residue levels between replicates. Therefore only results from the 50% soil dilution are presented. The correlation between white-rot growth quotient and PCP residue increased with time since inoculation, but never achieved statistical significance ($P>0.05$). This confirms earlier findings in Experiment 4 and by Boyd-Wilson et al. (2005 – Chapter 6) that colonisation of soil is isolate dependent. Figure 7.4 shows the fungal growth and PCP residue for each isolate after 168 days of incubation.

The rate of PCP decline and the final PCP residue level was affected ($P<0.001$) by the fungal isolate (Figure 7.5). The white-rot isolates fell into two groups: Isolates HR122, HR131 and HR577 with higher ($P<0.001$) PCP residues than isolates HR112, HR160 and HR590, but the latter were not different ($P>0.05$) from the control treatment (autoclaved SCSf). The control treatment, for example, had a final residue of 200 mg/kg PCP (standard error = 90) with a calculated decline of 5.32 mg PCP per kg soil per day (standard error = 0.74). The variance between replicates within isolate and sampling date was high, and similar to the variance among isolates as well as among isolates within sampling dates. This explains the relatively high standard errors observed. These variations in residue levels between samples are inherent when working with aged residues (EPA, 1992). Sampling and residue analysis was thus altered to reduce variation between replicates, as further described in Experiment 5b.

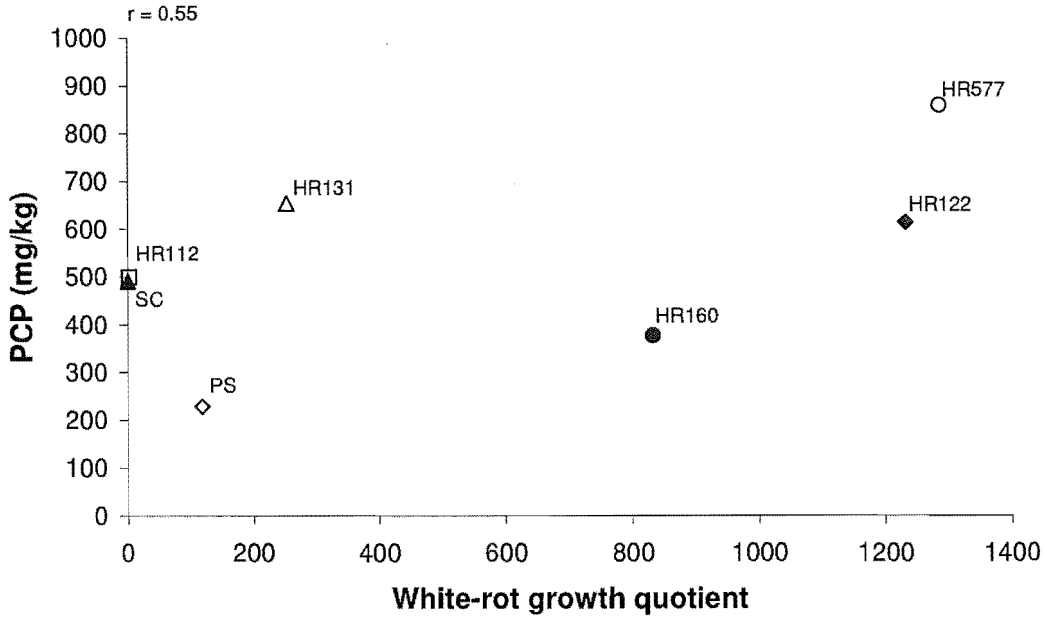

Figure 7.4. Mean PCP residue levels (mg/kg) after 168 days plotted against white-rot growth quotient (calculated area under the plot of white-rot cover) for each isolate. The correlation coefficient is given at the top left of the scatterplot. PS= *P. sordida*, SC = autoclaved SCSf.

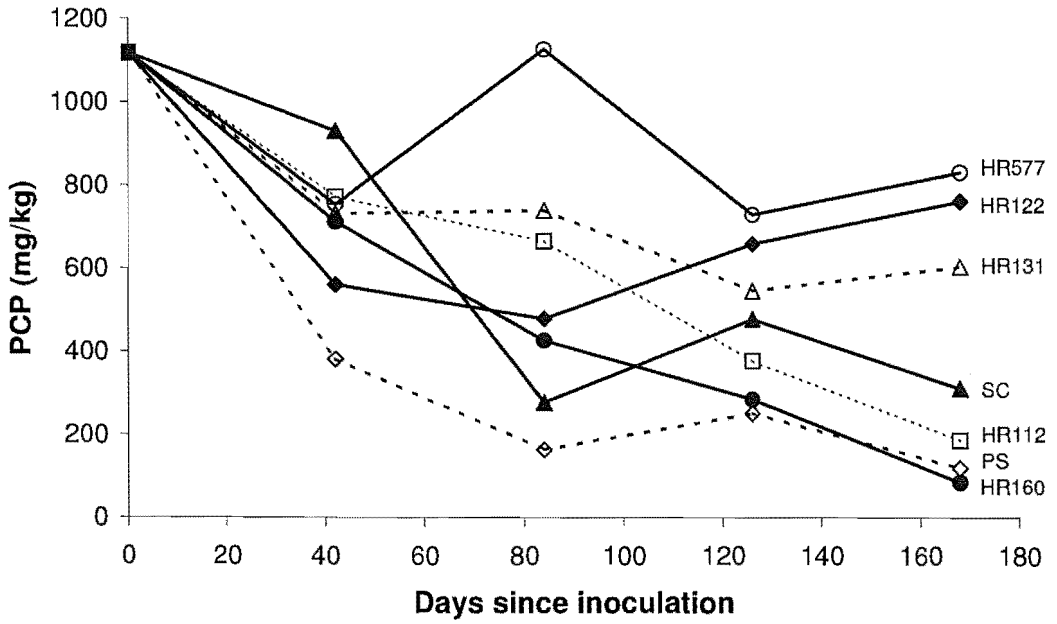

Figure 7.5. Mean PCP residue for each isolate plotted against the number of days since inoculation. PS= *P. sordida*, SC = autoclaved SCSf.

Although, PCP decline was affected by isolate treatment, PCP decline was not improved by inoculation with live (isolate HR160) versus dead mycelium (autoclaved SCSf isolate HR160). In earlier research, Walter et al. (2004 – Chapter 3) found that augmenting aged PCP-soil with arable top soil and autoclaved fungal inoculum resulted in a similar decline of PCP to augmentation with top soil and live inoculum, but live SCSf was required for mineralisation of PCP. In the current research, radiolabelled PCP was not used for reasons outlined in the introduction.

Treatment with autoclaved SCSf resulted in a significantly higher PCP decline than inoculation with three live fungal isolates. The autoclaved SCSf will serve as a nutrient source for the indigenous (PCP-soil) as well as augmented (top soil) microorganisms. Thus the autoclaved SCSf control could be considered a 'nutrient' treatment in its own right. Soil bacteria and fungi are known to degrade PCP (co)metabolically as reviewed by McAllister et al. (1996). Using live fungal inoculum, the nutrients augmented as part of the SCSf will be less accessible to other organisms. In addition, the live white-rot fungal mycelium will not provide a food source for soil microorganims, unlike in the autoclaved control, where growth substrate destructed by heat and pressure as well as the killed fungal mycelium will provide a readily accessible nutrient source.

Experiment 5b

In this small nested experiment, mean PCP residue values obtained are very similar between the two sampling methods (analysing 5 individual replicate specimen jars from a treatment *vs* the corresponding composite sample in duplicate). However, the variation is greatly reduced using the composite sampling method (Figure 7.6). Table 7.2 shows the corresponding (actual) PCP residue values including residue data for additional composite samples.

This experiment showed that the variation of the mean is greatly improved by PCP residue analysis using a composite sample. Generally, composite sampling is best applied to relatively uniform samples (EPA, 1992). At the set-up of experiment 5 and 5b, double sieving, and the use of a manual concrete mixer were employed to improve soil-pollutant homogeneity and ensure similarity between replicate samples. After treatment and incubation, uniformity of the composite sample was further ensured by thorough mixing and sieving of the combined samples. This is in agreement with EPA

recommendations (EPA, 1992). The subsequent reduction in variation (Table 7.2) is an indication of the overall improved sample-homogeneity.

Table 7.2. PCP residues (mg/kg) for Individual (Ind. = 5 individal replicates) and Composite (Comp. = duplicate residue analysis combining and homogenising 5 individual replicates) sampling methods for aged PCP residues augmented with top soil and white-rot fungi after 168 days incubation at room temperature

	PCP residues (mg/kg) for samples							
	(1)		**(2)**		**(7)**	**(11)**	**(15)**	**(25)**
Replicate	**Ind.**	**Comp.**	**Ind.**	**Comp.**	**Composite**			
1	150	399	3573	3361	751	554	296	3854
2	258	353	3452	3743	563	535	271	3192
3	602		3688					
4	708		1687					
5	54		2598					
Mean	354	376	3000	3552	657	544	283	3523
SD	286	33	850	270	132	13	17	468

Sample (1) = PCP-soil augmented with top soil (50%) and SCSf isolate HR112

Sample (2) = PCP-soil augmented only with SCSf isolate HR160

Sample (7) = PCP-soil augmented with top soil (50%) and SCSf isolate HR122

Sample (11) = PCP-soil augmented with top soil (50%) and SCSf isolate HR131

Sample (15) = PCP-soil augmented with top soil (50%) and SCSf isolate HR160

Sample (25) = PCP-soil augmented only with autoclaved SCSf isolate HR160

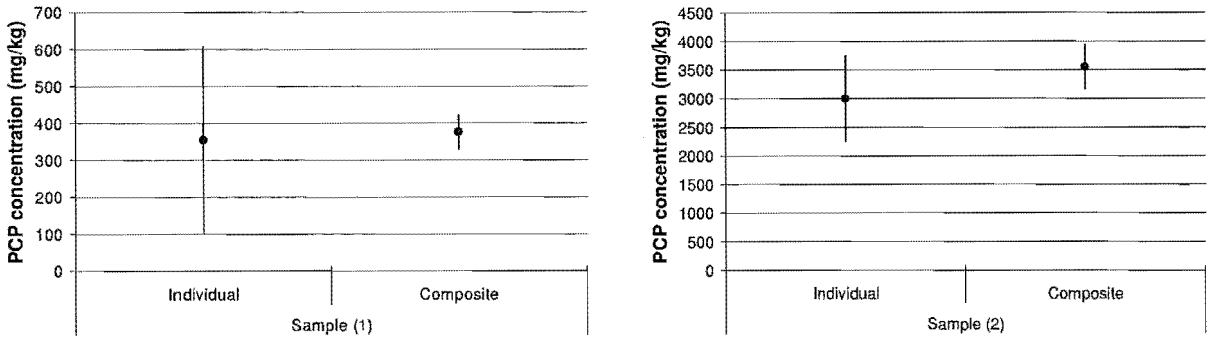

Figure 7.6. Average PCP (mg/kg) levels and confidence interval (95%) for the Individual (5 replicates) and corresponding Composite (duplicates) sampling methods after 168 days incubation at room temperature. Sample (1) was PCP-soil augmented with top soil (50%) and SCSf isolate HR112, and Sample (2) was PCP-soil augmented only with SCSf isolate HR160.

Conclusion

Aged PCP residues in soils are more toxic to white-rot fungi than an equivalent spiked PCP contamination. Pollutant degradation follows different patterns depending on whether the contaminant is aged or added. Therefore it is paramount to conduct bioremediation research on the actual polluted substrate. The upper limit for PCP bioremediation by New Zealand white-rot fungi seems to be in the order of 1300-1800 mg/kg PCP. Thresholds will probably vary between isolates and soil types.

Colonisation of soils by white-rot fungi is required to ensure pollutant degradation throughout the substrate. It is important to realise that colonisation itself is not an indication of PCP degradation, but a pre-requisite to ensure the organism (mycelium) meets the pollutant. Fungal isolates vary in their ability to colonise a particular soil/substrate, and also in their ability to degrade aged PCP resides in a particular soil/substrate.

This study recommends the use of composite sampling to give and estimate of the average PCP degradation in each tes cell. The use of composite sampling greatly reduces the number of analyses and can provide an accurate estimate of the average estimate of analyses (see experiment 5b) if subsampling is conducted without bias. The mixing of subsamples in composite sampling means that variability in performance

cannot be analysed to the same degree. In this case, the extra costs associated with PCP analysis meant that composite sampling was necessary.

Acknowledgements

We thank the Foundation for Research, Science and Technology for funding. We would also like to thank Drs Grant Northcott and Peter Alspach for scientific input relating to chemical residue analysis and statistical analysis, respectively.

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Chapter 8

Field-scale bioremediation of PCP using New Zealand white-rot fungi

Foreword

Chapter 8 describes the scale-up of fungal bioremediation based on the previous laboratory results. Leadership and management were conducted by M. Walter. However, for the proof-of-concept field remediation, guidance was provided to the student in various ways: D. McFadden, formerly of Woodward Clyde, helped with the design of the soil cells, with R. Chong and C. Ford, Massey University, providing the fungal inoculum. L. Boul, WRONZ helped with the inoculation of the soil cells and residue sampling, as well as being contracted to conduct the PCP residue analysis (TELARC accredited analytical laboratory). Analytical procedure for PCP residue monitoring is given in Appendix Chemical Residue Analysis. The soil cells, and materials thereof, were organised by and built by M. Walter, with help from M. Bürger. Dataloggers were set up by the student and the soil cells were monitored and the site maintained by M. Walter. Construction of the soil cells and preparation of the field site was conducted over a 3-month period, while the soil treatments were monitored and the site was maintained for a 3-year period by the student. Statistical analyses were

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Modified publication by

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Submitted to International Biodeterioration and Biodegradation, December 2004, accepted March 2005.

conducted by M. Walter. PCP residue data are given in Appendix to Chapter 8. A slightly modified version of the chapter below, has been submitted for publication by International Biodeterioration and Biodegradation.

Abstract

Engineered soil cells were designed to develop proof-of-concept biopiles for white-rot bioremediation of aged PCP contaminated soil from a former timber treatment site. Soil cells were constructed to allow for forced aeration, irrigation, leachate collection and monitoring of temperature and soil humidity. Parameters studied were the effect of a New Zealand *Trametes versicolor* isolate on PCP degradation, the effect of fungal inoculum concentration on PCP degradation and reproducibility of the experiments. PCP degradation and fungal survival were monitored at regular intervals for 2.5 years. The experiments were set up in January 2000. There was no effect of inoculum concentration, and treatment effects were reproducible. PCP residue levels declined from 800-1000 mg/kg to 4 (0-9.4 mg/kg) in a first order kinetics. Irrigation was not required during the 2.5 years of the study, nor did leachate form. The soil cells did not exceed average daily temperatures of 35°C. The results support the conclusion that an isolate of the New Zealand white-rot fungus *T.versicolor* can biodegrade PCP from aged soils in a field situation.

Keywords: Field remediation, biopile design, PCP, *Trametes versicolor*

Introduction

In New Zealand, pentachlorophenol (PCP) was used extensively by the forestry industry to prevent sapstaining of wood. An estimated 5000 tonnes of PCP was used at approximately 800 timber treatment sites throughout New Zealand over a period of approximately 40 years (Anon., 1995). PCP was finally banned in 1991 (Finnbogason and Quintin, 1994). The widespread use of this chemical resulted in a variety of waste streams, including contaminated soils from drippage and spills.

White-rot fungi have been shown to degrade a wide variety of environmental pollutants including PCP (Pointing, 2001). Degradation of PCP by the American fungi *Phanerochaete chrysosporium* Burds. and *Phanerochaete sordida* (Karst.) Erikss. &

Ryv. has received particular attention (Mileski et al., 1988; Lamar et al., 1990a, 1990b; Lamar and Dietrich, 1990, 1992; Lamar et al., 1993). However, other white-rot fungi, such as *Trametes versicolor*, have shown potential as PCP degraders (Roy-Arcand and Archibald, 1991; Seigle-Murandi et al., 1991, 1993; Alleman et al., 1992; Lamar and Dietrich, 1992; Ricotta et al., 1996). Therefore the potential of New Zealand native white-rot fungi for bioremediation of PCP polluted soil sites was investigated (Walter et al., 2003 – Chapter 2). Mineralisation studies (Walter et al., 2004 – Chapter 3) showed that in liquid culture mineralisation rate was higher for New Zealand *Trametes versicolor* (L.: Fr.) Pilat isolates compared to the American isolate of *P. chrysoporium.* Very little to no pentachloroanisole (PCA) was captured in the volatile fraction of the *T. versicolor* isolates, whereas 75% of the volatile fraction of *P. chrysosporium* consited of PCA (Walter et al., 2004 – Chapter 3). In soil microcosm studies with contaminated soil (200 mg/kg PCP) from a timber treatment site, the New Zealand *T. versicolor* isolates also mineralized PCP. Approximately 34% of initial radioactivity added was captured in the CO_2 fraction (Walter et al., 2004 – Chapter 3).

The objective of this research was to further evaluate PCP bioremediation in the field for one selected *T. versicolor* isolate. Two field trials were conducted, with the second field trial still ongoing.

Materials and methods

Fungus and inoculum preparation

The *T. versicolor* isolate HR131, deposited as Culture A at the Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia with Accession number NM02/27875, was maintained as mycelial plugs in 7 mL bijou bottles (Samco Laboratories) of sterile distilled water at room temperature in the dark. The fungus was grown by transferring a mycelial plug, from the bijou bottle onto a malt extract agar (MEA, Merck) plate. Plates were incubated in the dark at 25-30°C for 7-21 days. Fungal inoculum for the field remediation was produced in two steps, first at a 4 L scale production (henceforward referred to as spawn) and then in 500 L batches (henceforward referred to as field inoculum). Both spawn and field inoculum were based on a sawdust-cornmeal-starch (SCS) formulation as described by Leštan and Lamar (1996).

The spawn was prepared by inoculating 4 L of sterilised SCS (50% water w/w) with fungal mycelium of HR131. SCS was placed in autoclave bags (6 L capacity, Biolab Scientific) fitted with an aluminium tube (50 mm diameter x 70 mm height), which in turn was sealed with non-absorbant cotton wool or a foam plug. The SCS bag was autoclaved twice (110 kPa, 121°C) for 1 hour with a 24 h interval. The fungal mycelium was produced by aseptically inoculating 100 mL sterile malt extract broth (Merck) in 250 mL Erlenmeyer flasks with four mycelial plugs (6 mm diameter) of a 7 day old MEA plate of isolate HR131. A sterile glass bead (25 mm) was added to each flask. Flasks were incubated on an orbital shaker (100 rpm) at room temperature (20-25°C). The glass beads caused growth of small (<3 mm) mycelial 'balls'. After 7 days growth, the whole content of an Erlenmeyer flask was poured aseptically into a sterilised spawn bag and incubated in the dark at 25°C for a further 36 days.

For the field trial the field inoculum was produced in a glasshouse at Palmerston North in 500 L batches of SCS as follows. SCS (50% water w/w) was spread (200-300 mm high) on a ground cover (50 μm high density polyethylene (HDPE), PlaceMakers) and sterilised with a fumigant (Basamid®, Yates) according to the manufacturer's instructions. The pile was covered with a HPDE sheet and the pile was turned every 3-5 days for 36 days to dissipate the fumigant gases. After 36 days the SCS pile was inoculated with the fungal spawn (4 spawn bags/500 L field inoculum) by manually breaking the well-colonised and leathery spawn into pieces (< 100 mm) and mixing into the fumigated SCS. The field inoculum pile remained covered up and was sampled at regular intervals to determine the optimum time for inoculation of the polluted soil. This was based on the chloride release potential according to the protocols by Walter et al. (2004 – Chapter 3). The field inoculum was deemed ready for use once there was greater than 15% theoretical chloride release which was after approximately 90 days.

Engineered soil cells

Soil cells were designed to be proof-of-concept biopiles for white-rot bioremediation of aged PCP contaminated soil from a commercial timber facility. The soil cells were constructed to allow for forced aeration, irrigation, leachate collection and monitoring of temperature and soil humidity. A schematic layout and design of the soil cells is given in Figures 8.1-8.3 and Figure 8.4 depicts the soil cells after construction and inoculation. Up to three temperature and three moisture sensors were

placed in the centre of each cell at different heights (approximately 100-150 mm spacing between each level of probes). Temperature was measured using thermistor temperature probes and soil (inoculum-mix) moisture was measured using water content reflectometers (CS615, Campbell Scientific). Data were recorded at 10 min intervals using a CR10X datalogger equipped with a ADM-AO4 four channel analog output module (Campbell Scientific).

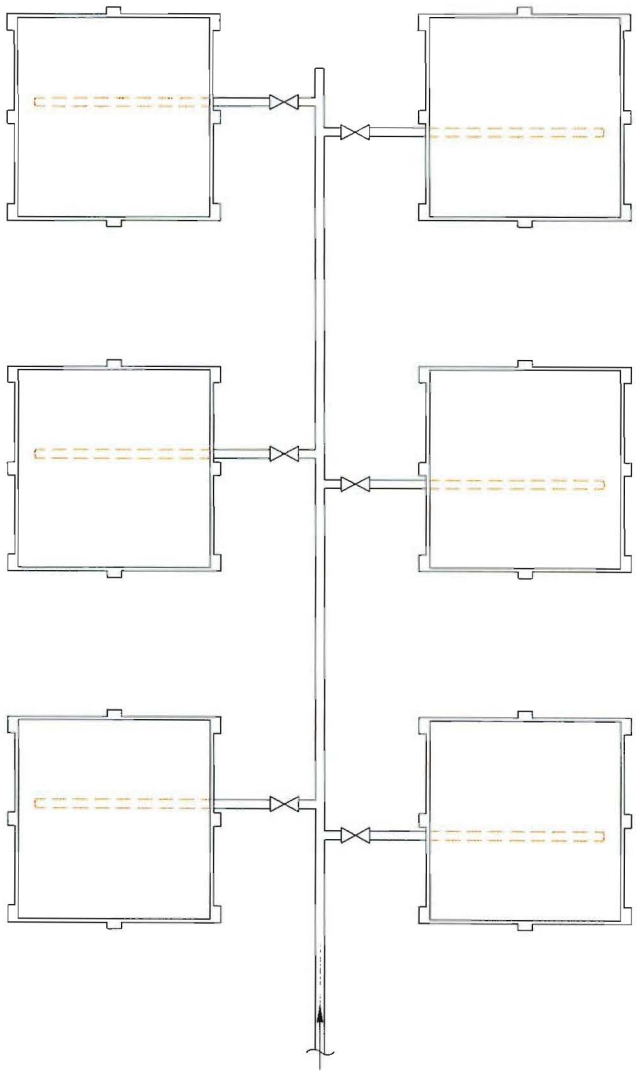

Figure 8.1. Schematic outline of the engineered soil cells. For detailed measurements please refer to Attachment 8.1. (Electronic drawings by James Pinfold)

Aeration pipes (PVC, 65 mm diameter, Novapipe) were installed on the surface of the ground on river gravel as shown in Figures 8.2. Gate valves (32 mm diameter) were fitted for control of flow rate. All pipe materials and valves were obtained from Mico

Wakefield Ltd. In the soil cells, aeration pipes were embedded in river gravel (20 mm, with no fines). The gravel was covered with a filter fabric (Mico Wakefield Ltd). Aeration of the biopile was facilitated by ten, evenly spaced, 50 mm sawcuts wrapped with stainless tie wire along the embedded Novapipe (Figure 8.3). The stainless wires create small eddies within the pipe, facilitiating even airflow through the sawcuts (D. McFadden, pers. comm.).

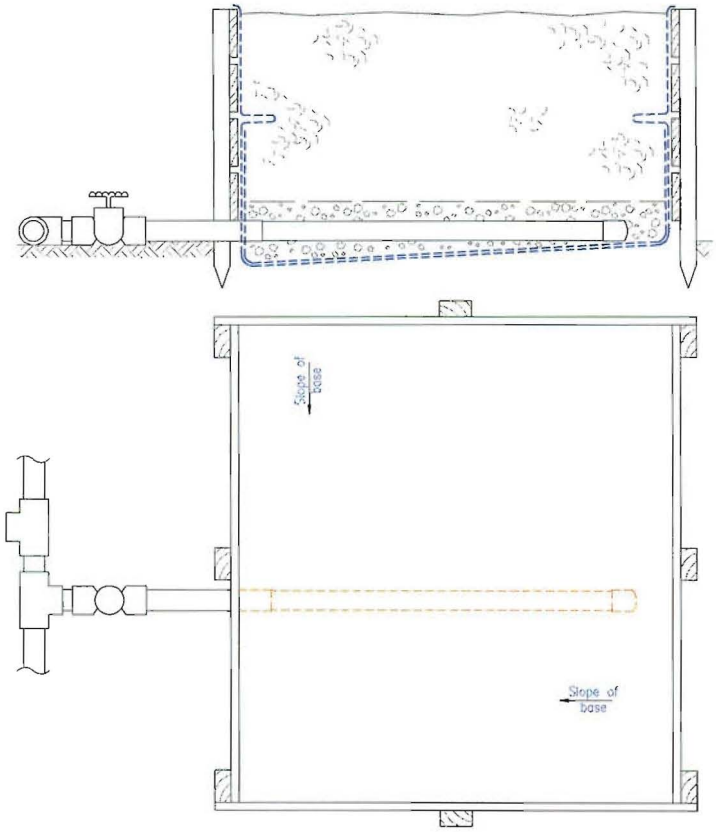

Figure 8.2. Cross section of soil cells. Note; the plastic liner is double on the bottom/gravel part of the pile with a mud guard between gravel and soil. For detailed measurements please refer to Attachment 8.1. (Electronic drawings by James Pinfold)

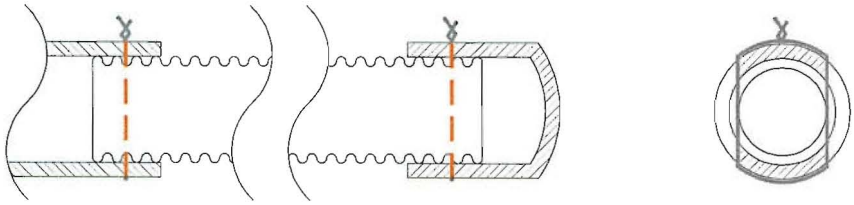

Figure 8.3. Detail of aeration pipe saw cuts and wire insert. For detailed measurements please refer to Attachment 8.1. (Electronic drawings by James Pinfold)

Soil cells were simultaneously aerated (gate valves fully opened) by blowing air through the biopiles for 30 min at 5 h intervals using a single phase fan unit (CR Elec HB 329, McEwings Pumping Systems Ltd). Based on the fan output, the calculated air flow was approximately 6 L/s to each unit, giving a theoretical velocity through the pile of approximately 3 mm/s.

Figure 8.4. Soil cells used in this study.

Inoculation of soil cells

Parameters studied were the effect of a selected white-rot fungus on PCP degradation (Soil Cells 1 and 2), reproducibility of the experiments with a different inoculum (Soil Cell 3) and the effect of fungal inoculum concentration on PCP degradation (Soil Cell 4). Fungal field inoculum (produced in 500 L batches as described above) was sent overnight from Palmerston North to the Canterbury field site in 200 L drums. A 200 L drum of aged PCP contaminated soil was obtained from a commercial mill in New Zealand. Soil contamination levels in the drum ranged from

15–35 g PCP/kg dry soil. The contamination was 35-40 years old. At inoculation, the sandy PCP contaminated soil was diluted with local screened top-soil (Temuka deep clay loam) by mixing 1 L aged PCP contaminated soil with 20 L of top-soil (this mixture is henceforward referred to as PCP-soil) in a concrete mixer. Willow (*Salix* spp.) wood chips (40-80 mm length x 10-20 mm diameter) were obtained from locally grown trees and shredded on site.

Two soil cells were inoculated on the 21st January 2000 and two more cells on the 15th March 2000. A concrete mixer was used to blend PCP-soil, wood chips and 50 L batches of field inoculum (henceforward referred to as inoculum-mix). The ratio (% volume) of soil:wood chips:field inoculum was 60:20:20, except for one cell (Soil Cell 4) inoculated on the 15th March 2000, where the final ratio of soil:wood chips:field inoculum was 40:20:40. After each 50 L batch, the inoculum-mix was spread evenly into the soil cell, approximately 5 L of water was sprinkled over the top to reach a final water content of approximately 60% moisture (w/w).

Soil cells were filled until 250 L field inoculum/soil cell was used up. Then the HPDE lining of the soil cells was folded into the centre and an additional HPDE sheet put on top covering the inoculum-mix to reduce excessive surface drying. Additionally, soil cells were equipped with a simple lid to prevent rain penetration (Figure 8.4). Treatment descriptions for the different soil cells are summarised in Table 8.1.

Table 8.1. Biopile treatments

	Soil Cell 1	**Soil Cell 2**	**Soil Cell 3**	**Soil Cell 4**
Fungal Inoculum (%)	20	20	20	40
Willow wood chips (%)	20	20	20	20
PCP-soil (%)	60	60	60	40
Date of inoculation	21.1.00	21.1.00	20.3.00	20.3.00

PCP degradation and residue analysis

For monitoring PCP degradation, inoculum-mix samples were initially taken weekly followed by progressively longer intervals (1, 2, 3, 4, 5, 8, 10, 12, 16, 20, 44, 52, 70 and/or 78 weeks after inoculation). Sampling consisted of a single core taken with a 50 mm diameter auger style corer. The core was taken through the entire depth of the pile to the gravel base. After sampling, the resulting hole was filled with screened soil and the sampling location labelled with a tag. Soil cells were emptied in November 2001 for a second field trial. All inoculum-mix from all soil cells was removed and piled into a container, which was covered with HDPE sheeting to prevent rain penetration. However, the PCP decline was still monitored by collecting 4 replicate core samples with the auger in August 2002 and August 2003.

The total soil sample was sieved (2 mm mesh), collecting all soil that freely passed through. Stones and wood chips were not included in the analysis and results are expressed on a dry weight basis of the sievable portion. Sieved soils were sub-sampled and moisture contents determined by oven drying a 10.0±0.1 g (fresh weight) sample at 105°C for 24 h. Soil (10.0±0.1 g fresh weigh) was extracted with 20 mL extraction solution (50:50 acetone:hexane, saturated with 2.5 M HCl) in a 50 mL Teflon centrifuge tube by end-over-end shaking for 1 h, or by ultrasonication for 30 min. After extraction, the tubes were centrifuged and the supernatant transferred to a 100 mL separating funnel containing 20 mL of wash solution (50:50 0.2M HCl:saturated NaCl).

The pellet was similarly extracted twice more with 10 mL extraction solution and shaking for 10 min, and the supernatants pooled. The separating funnel was shaken to wash the hexane layer, and the aqueous phase discarded. The organic phase was then washed again against a further 20 mL wash solution, which was then discarded. The

organic phase was then passed through an ALLTECH 500 mg Maxiclean silica SPE cartridge hand packed full with anhydrous Na_2SO_4 (approximately 2 g) at a flow of around 2 mL/min, and the eluate collected into a 50 mL volumetric flask. The funnel was rinsed with 5 mL hexane, before passing this through the cartridge. After the hexane extract had passed through the cartridge, the reservoir and the SPE were rinsed with 5 mL 50:50 dichloromethane:hexane, collecting all rinsings into the volumetric flasks. These were then made to 50 mL with hexane. Two aliquots of approximately 1 mL each were transferred to GC vials and capped with Teflon septa ready for GCMS SIM analysis (PCPSIM3.MET).

Statistical analysis

All statistical analysis was conducted using Minitab Version 12.1. Analysis of Variance (ANOVA) was used to determine the effect of inoculation time and fungal inoculum concentration on PCP residue levels. Regression analysis was used to describe PCP decline over time. Correlation analysis was conducted to determine the relationship of moisture and temperature probes between and within the soil cells.

Results

There was no statistically significant difference ($P>0.05$ that the null hypothesis holds) in PCP levels (when expressed in equivalent weeks after inoculation) between the 4 soil cells, irrespective of inoculation time and fungal inoculum. Therefore the individual soil cells were used as replicates in the further regression analysis of the PCP residue data.

Field remediation using HR131 reduced aged PCP residue from approximately 800 mg/kg to 100 mg/kg within one year of treatment and to less than 50 mg/kg after 74 weeks (Figure 8.5). After the initial decay, PCP concentration was gradually declining during the period monitored. There was a high variation in PCP levels between replicate soil cells and sampling times. The variation of PCP levels within a sample core from a soil cell was also relatively high. However, over time PCP levels declined exponentially (Figure 8.5).

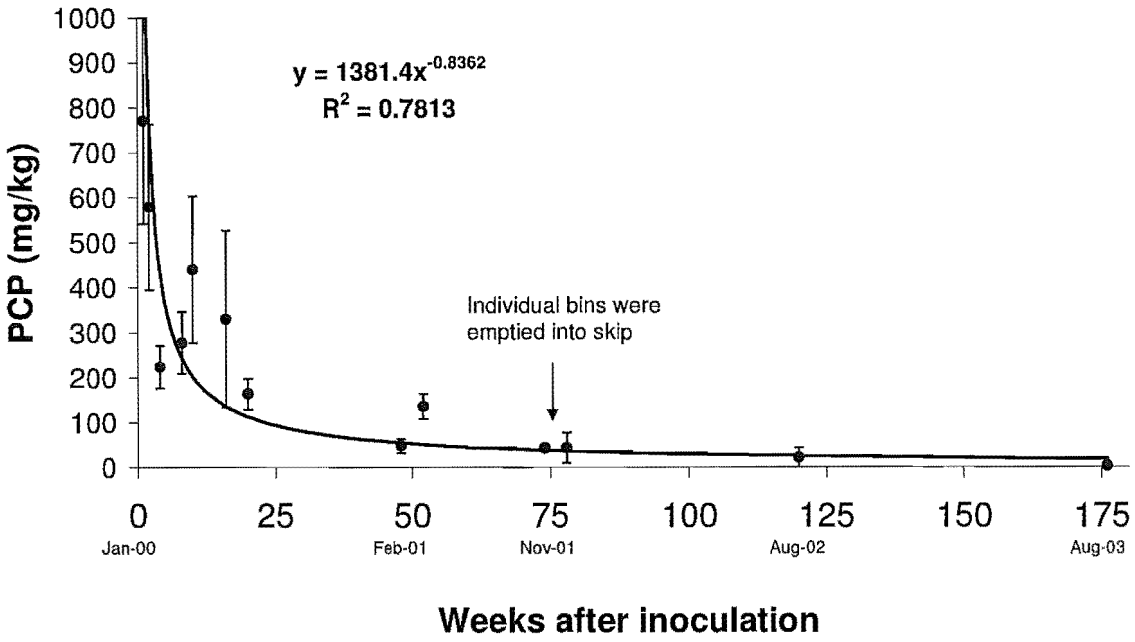

Figure 8.5. Average decline in PCP contaminated soil in the field after inoculation with the New Zealand white-rot fungus *T. versicolor* HR131. Each point consists of the mean for 2-4 soil cells. Error bars indicate the standard deviation of the menas.

Mean daily temperature and moisture readings are shown for Soil Cell 1 in Figure 8.6. Irrigation was not required during the duration of the experiment because soil moisture increased during treatment. This was most likely due to metabolic water from decay of woody material. However moisture increase was gradual and no leachate was collected. Temperatures within the soil cells were largely determined by the outside temperature. Both, temperature and soil moisture measurements were highly correlated within a soil cell ($r>0.9$; $P<0.001$) and between soil cells ($r>0.9$; $P<0.001$).

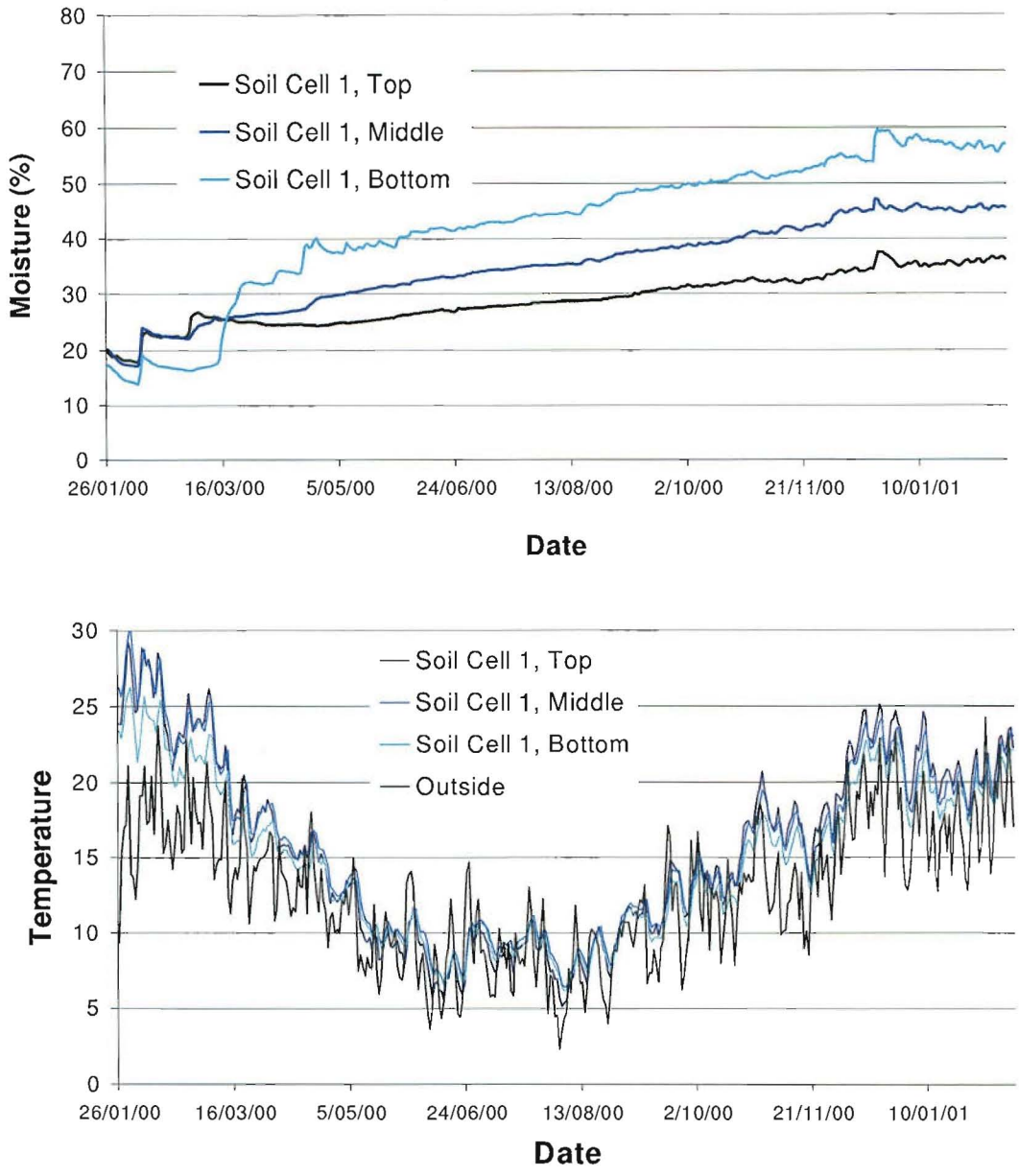

Figure 8.6. Daily average measurements for soil moisture and temperature (°C) for Soil Cell 1 from 26 January 2000 to 16 February 2001 for probes positioned at the bottom, centre or top of the soil cell.

Observing the soil cells, a mycelial flush occurred within two weeks of inoculation (Figure 8.7). The woodchips added were completely decomposed within 8 months.

Figure 8.7. Mycelial flush (Soil Cell 1) 2 weeks after inoculation with the white-rot fungus. A NZ$1 coin (22 mm diameter) is shown.

Discussion and conclusions

It is clear that the PCP residue data were rather variable. This largely reflects heterogeneity within the bin, as extraction efficiencies of spiked samples analysed using the method were close to 100%. The variability was observed over small distances as samples analysed with five replicates showed high variation. This variability is possibly due to the method of PCP addition to the soil cells. A very sandy soil with high PCP was added at approximately 1:30 dilution. While this potentially produced a reasonable distribution of PCP within the soil cell, if the PCP is immobile in the soil cell, the 10 g subsamples used for extraction, may have varied in the number of discrete PCP contaminated particles present. This also explains the variation within samples and sampling times. Further, the distribution of air was not tested among and within soil cells. Aeration and the amount of available oxygen may affect the PCP-fate in soil environments. However, notwithstanding these variations, PCP levels in the treated soil cells declined.

PCP decline may have been supported by the mixing of PCP contaminated soil with local top soil and thus augmenting with soil microrganisms potentially able to degrade PCP (McAllister et al., 1996). In earlier work, we (Walter et al., 2004 – Chapter 3) showed that PCP degradation also occurred in the absence of white-rot fungi by diluting contaminated soil with topsoil. However, PCP mineralisation only was observed in the presence of live white-rot fungi and PCP decline was greater in soil with white-rot fungi than without white-rot fungi.

The temperature measurements clearly showed that biogenic heat was not produced. The biopiles did not generate their own heat as occurs during composting processes. Equally, despite the aeration, the soil cells did not dry. Moisture is important for fungal colonisation (Walter et al., 2005 – Chapter 5), while temperatures exceeding 40°C can kill the *T.versicolor* inoculum (Walter et al., 2003 – Chapter 2).

Previous (Walter et al., 2003; 2004 – Chapters 2; 3) research has shown that *T. versicolor* can decompose PCP in liquid and in soil. This earlier laboratory based research was successfully applied in the field. This is similar to other research findings. Remediation of PCP in the field by other white-rot fungi was successfully demonstrated, for example, for *P. chrysosporium* by Holroyd and Caunt (1995); and for *P. chrysosporium, P. sordida* and *T. hirsuta* by Glaser and Lamar (1995).

P. chrysosporium is considered a model organism for PCP degradation (Ricotta et al., 1996), however *T. versicolor* may be equal or superior to *P.chrysosporium. T. versicolor* displayed higher tolerance to PCP (Alleman et al., 1992; Walter et al., 2003 – Chapter 2) and showed better degradation in soil (Boyle, 1995; Leštan and Lamar, 1996) and liquid culture (Walter et al., 2003 – Chapter 2). Further, unlike *P. chrysosporium, T. versicolor* produces negligible amounts of PCA during PCP degradation (Tuomela et al., 1999; Ullah et al., 2000; Walter et al., 2004 – Chapter 3).

Acknowledgements

We would like to thank the Foundation for Research, Science and Technology for funding. Thanks to Manfred Bürger for help in building the soil cells and to Environment Canterbury for providing the research site. Special thanks to Mike Hyatt and his colleagues (Environment Canterbury) for kindly providing us with fresh willow wood. Thanks also to Fletcher Challenge for supplying contaminated soil. Thanks to James Pinfold for producing the electronic drawings of the soil cell layout and design.

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Attachment 8.1.

Detailed measurements for Figures 8.1.to 8.3 on subsequent folded insert (A3-format)

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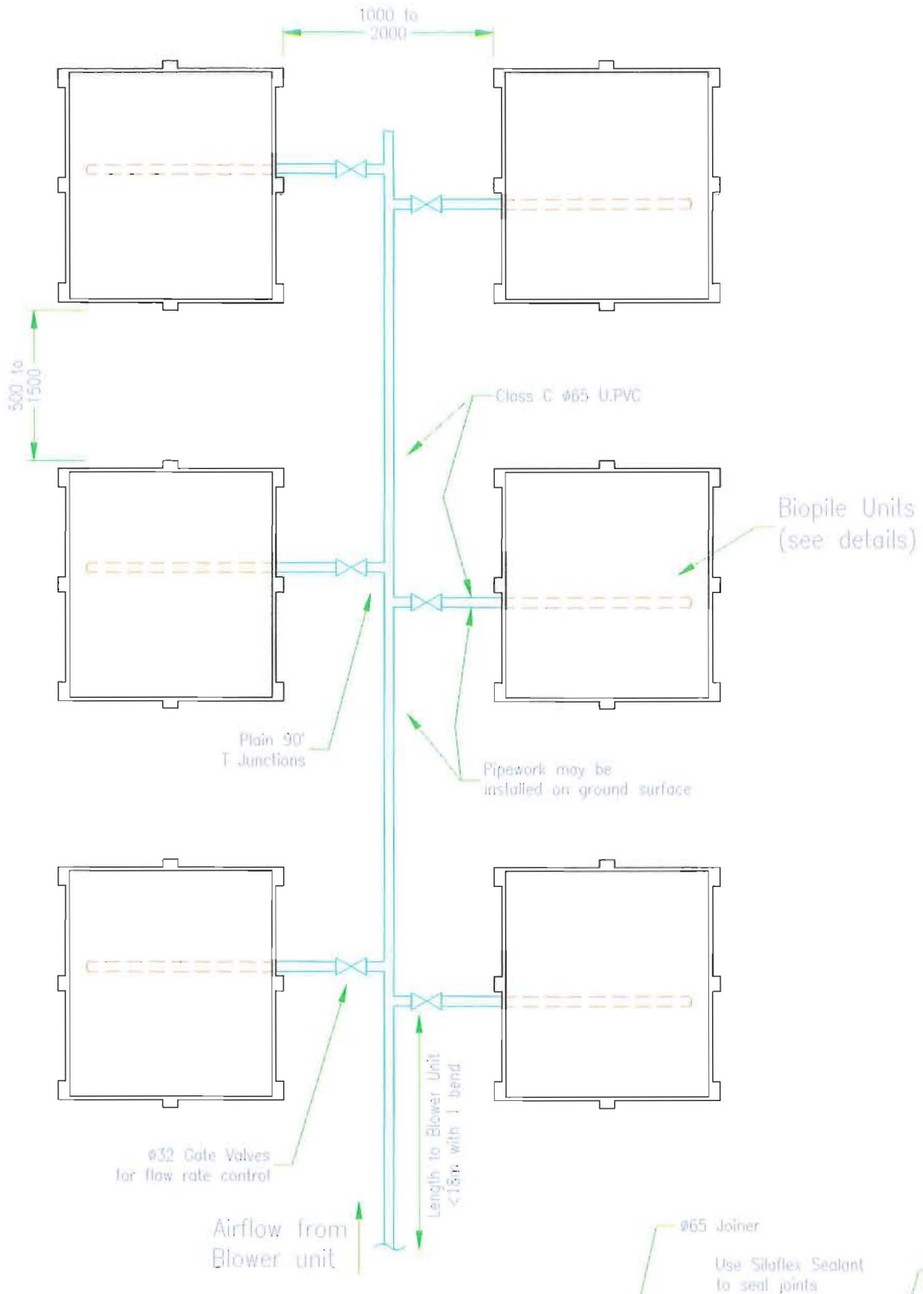

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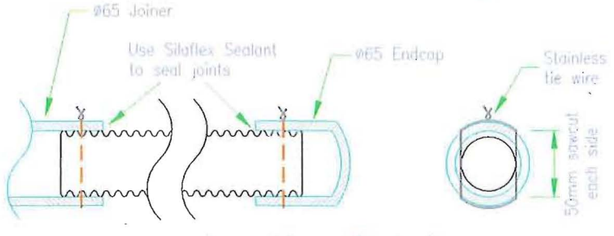

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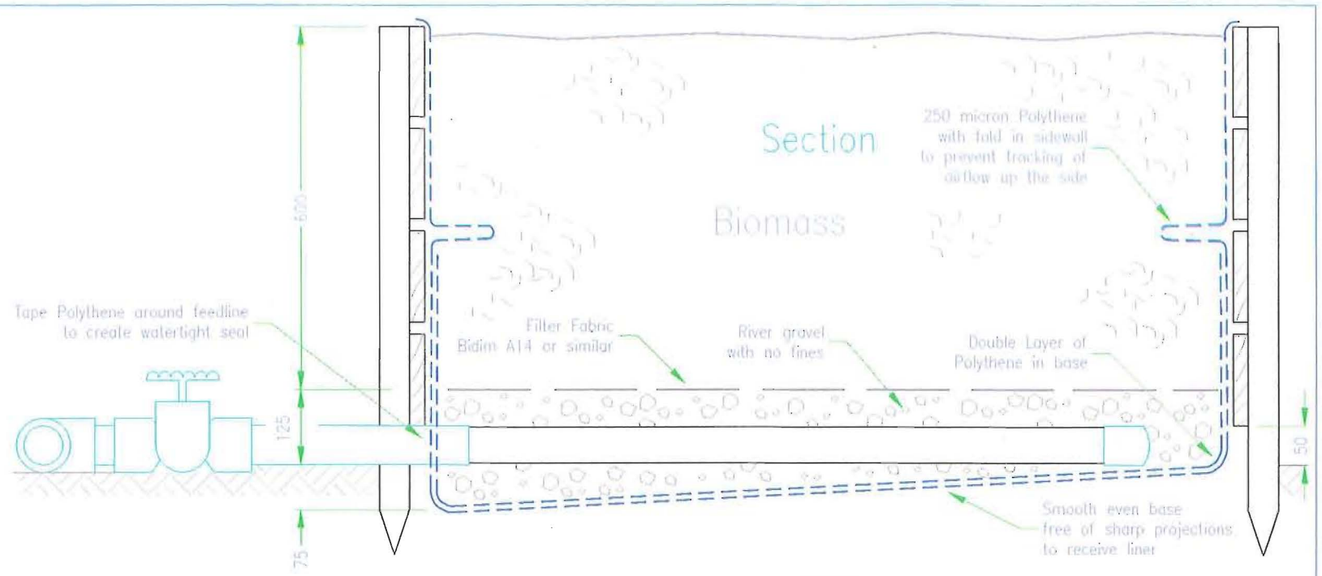

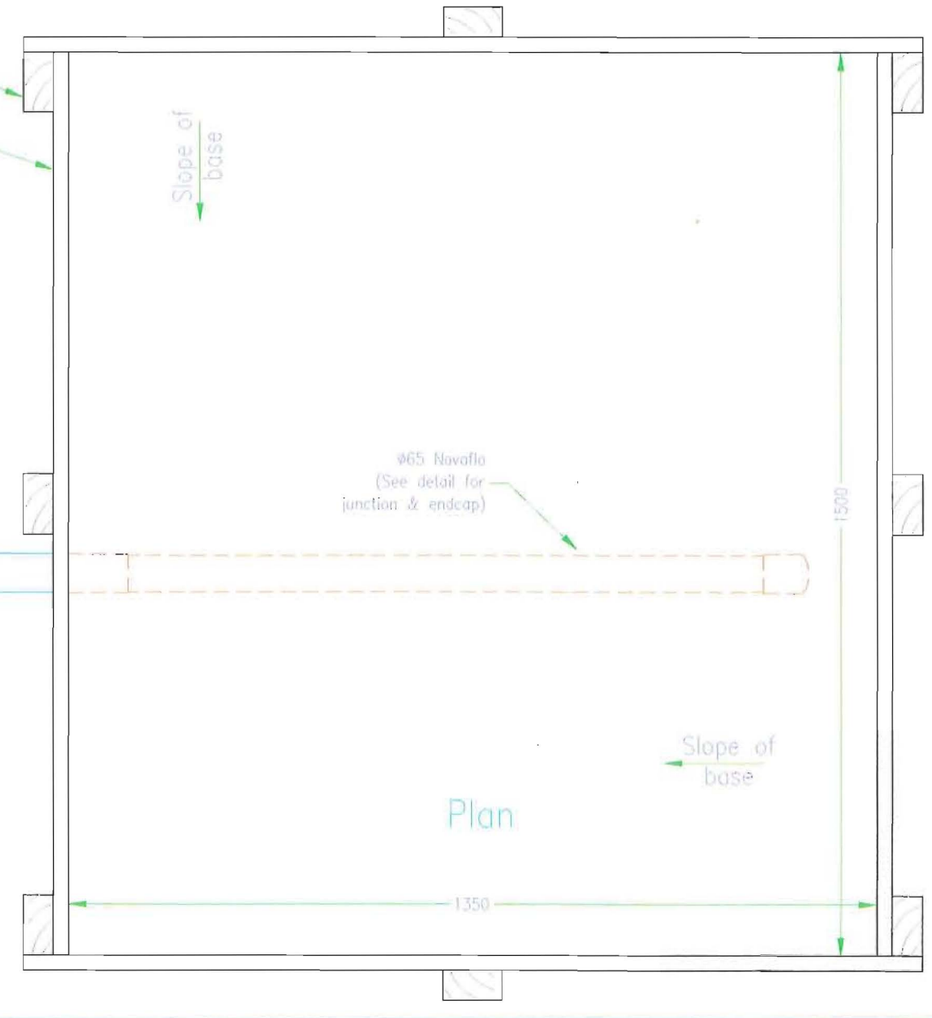

Plan

AMENDMENTS	BY	APPD.	DATE

	NAME	DATE
ACAD NAME	Biopile	021105
DESIGN BY	Monika Walter	
DRAWN BY	J Pinfold	
APPROVED		

Hort+Research
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Layout & Details

SCALE: 1:10	ACAD SCALE:	ISSUE:
JOB:	PAPER SIZE: A3	SHEET No:
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Chapter 9

Summary: Development of a biotechnology tool using New Zealand white-rot fungi to degrade PCP

Foreword

Chapter 9 is a summary and overview of the research results presented in the previous chapters and adds additional information as appropriate. Chapter 9 describes the development of a white-rot fungal bioremediation technology from the onset of screening isolates to the application of bioaugmentation in the field. A modified version of the chapter below, has been submitted for publication in the Conference Proceedings book of the BioMicroworld2005 Symposium, 14-18 March 2005, Badajoz, Spain.

Abstract

Research on the development of a biotechnology tool using New Zealand native white-rot fungi to degrade pentachlorophenol (PCP) is reported. Studies focused on the selection of isolates with degradation potential as well as building a database on growth and survival characteristics, prediction of biodegradation potential, and the transfer of

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Modified publication(s) by

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Modified versions presented orally at the WasteMINZ Conference, Rotorua 2002, and SETAC Conference, Christchurch 2003.

Development of a biotechnology tool using New Zealand white-rot fungi for bioremediation of pentachlorophenol – a summary. Submitted for publication in March 2005 as a review paper in conjunction with the the BioMicroworld2005 Symposium, 14-18 March 2005, Badajoz, Spain.

protocols to the field. Field remediation using a superior white-rot isolate reduced 800 mg/kg aged-PCP residue to 50 mg/kg over a 74 week period. Continued incubation reduced PCP residue levels to below 5 mg/kg.
Keywords: *Trametes versicolor*, bioremediation, PCP, laboratory studies, field evaluation.

Introduction

White-rot fungi are a physiological rather than a taxonomic grouping of fungi, so called because of the bleached appearance of the wood attacked by these fungi. White-rot fungi degrade lignin in order to access wood polysaccharides locked in lignin-carbohydrate complexes. Lignin is extremely recalcitrant and is only degraded by white-rot fungi such as *Trametes versicolor* (L.: Fr.) Pilat. In order to degrade lignin, white-rot fungi excrete one, or more, extracellular enzymes which are non-specific. These enzymes have also been shown to degrade a wide variety of environmental pollutants (Pointing, 2001), including pentachlorophenol (PCP) (Mileski et al., 1988; Lamar et al., 1993; Logan et al., 1994; Tuomela et al., 1999; Walter et al., 2003; 2004 – Chapters 2; 3).

Before it was banned in 1991, PCP was used extensively by the forestry industry to prevent sapstain of wood. An estimated 5000 tonnes of PCP was used (Finnbogason and St Quintin, 1994) at approximately 800 timber treatment sites throughout New Zealand, over a period of approximately 40 years (Anon., 1995). This widespread use of the chemical resulted in a variety of waste streams, including contaminated soils from drippage and spills.

Because of restrictions on the importation and use of overseas micro-organisms our research has focused on the use of New Zealand white-rot fungi to degrade PCP contaminated soils. Research focused on the selection of isolates with degradation potential as well as building a database of information on growth and survival characteristics, prediction of this potential and the transfer of protocols to the field. This paper reviews research carried out over the past 7 years by the "white-rot bioremediation team" of the Environmental and Risk Management Group, HortResearch. The paper focuses on experimental work leading to a patent application No. 519022 in May 2002. The patent describes growth and survival studies as well as

the bioremediation potential in the laboratory, and the evaluation of PCP bioremediation in the field. This review does not include research conducted on formulation technology of specific white-rot isolates, nor the mechanisms and pathways of degradation, nor molecular approaches used for the detection and evaluation of fungal activity within the biopile. These will be reported elsewhere.

Materials and methods

Fungi and inoculum preparation

Isolates were obtained from bioprospecting, and as gifts from Landcare Research, Auckland, and Forest Research, Rotorua. A total of 481 isolates were collected from at least 77 different genera. Not all isolates could be identified. The two American isolates *Phanerochaete chrysosporium* (ATCC 24725) and/or *P. sordida* (ATCC 90628) were included as controls. Isolates were maintained as described by Walter et al. (2003 – Chapter 2). Inoculum for lab based experiments was prepared on agar, and on a fungal growth substrate (SCS) as described by Walter et al. (2003 – Chapter 2). Inoculum for the field remediation was produced in 500 litre batches of SCS. The remediation potential of the SCS inoculum was monitored using biological potential, laccase and ergosterol measurements and/or chloride release measurements (Walter et al., 2004 – Chapter 3).

Growth and survival studies

Temperature tolerance. Two hundred and sixty-one isolates were studied to determine their upper and lower temperature limits for growth as described by Walter et al. (2003 – Chapter 2). Growth on agar was measured after 3 and 7 days incubation at 0, 5, 20, 25, 30, 35 and 40°C. If little or no growth was observed during incubation, isolates were transferred to 25°C for 7 additional days to assess recovery of growth.

Ligninolytic activity

The production of the extracellular enzymes essential for lignin degradation and associated with degradation of pollutants was measured using polmeric dye decolorisation and by assessing wood decay. For the dye assay, 367 isolates were assessed for the presence or absence of discoloration according to the method of Glenn and Gold (1983). Wood decay of fresh willow cuttings was assessed for 235 isolates by

inoculating one end of the cutting with white-rot fungi from agar culture and incubating. The non-inoculated end of the cutting was then submerged in water at room temperature for 3 months. Stems were split longitudinally and the length of decay of wood measured (Walter et al., 2003 – Chapter 2).

Colonisation of New Zealand soil by white-rot Trametes versicolor *isolates.* The growth of three *T. versicolor* isolates in 11 New Zealand soils, chosen according to their geological make-up and age, was investigated. Correlation studies between soil properties and colonisation were also conducted (Boyd-Wilson et al., 2005 – Chapter 6). Briefly, SCS was mixed with each soil at a ratio 1:4 (by volume) and the mixture sterilised. After 7 days incubation at 30°C in the dark, the area covered and the density of mycelial growth was measured to give a colonisation index.

Growth of selected Trametes versicolor *isolates in PCP contaminated soil*

Aged-PCP residue soil was mixed with a soil containing no PCP and with SCS at a ratio of 3:1 (by volume) soil to SCS to give final theoretical concentrations of 0, 20, 50, 100 and 200 mg/kg PCP as described by Walter et al. (2005 – Chapter 5). The mixture was saturated and left to drain to reach container capacity. Fungal inoculum of 21 day old cultures of 3 *T. versicolor* isolates growing in glass petri dishes (100 mm), incubated at 30°C in the dark, was used to inoculate the mixture. The inoculum was turned out into a 2 L ice-cream container and 250 mL of soil-SCS mixture of the appropriate PCP concentration was placed around and to the top of the inoculum. The ice-cream containers were placed in plastic bags and incubated at 30°C in the dark.

At 7 and 14 days, the diameter of growth (mm) of white-rot mycelium and the density of mycelial growth was measured using a rating system: 1 – scattered hyphal growth, 3 -medium coverage of soil by hyphae at three times the density of 1 and 5 – dense coverage of soil by hyphae at five times the density of 1. The two assessments were then combined to form a colonisation index with colonisation = diameter x density (Walter et al., 2005 – Chapter 5).

Laboratory degradation potential studies

Tolerance to PCP in agar. The growth of 163 isolates, selected on genera and origin, on malt extract agar (MEA, Merck) amended with 0, 10, 20, 30, 40 and 50 mg/L

PCP was measured after 3, 7 and 14 days incubation at 25°C. An additional 90 isolates (including *P. chrysosporium* and *P. sordida*) were screened for PCP tolerance at 0 and 50 mg/L PCP. Isolates showing growth at 50 mg/L PCP were also tested at 100, 150 and/or 200 mg/L (Walter et al., 2003 – Chapter 2).

PCP degradation in liquid culture. Twenty isolates (Table 9.1) tolerant to PCP at 200 mg/L PCP in agar were tested for their ability to degrade PCP in liquid culture containing 50 mg/L PCP (Walter et al., 2003 – Chapter 2). After 42 days of static incubation at room temperature the filtrate was analysed for PCP by HPLC. The activity of the extracellular enzyme laccase was monitored at regular intervals during incubation (Walter et al., 2003 – Chapter 2).

Degradation of PCP in soil microcosms. The ability of 22 New Zealand white-rot isolates (Table 1) tolerant to 200 mg/kg PCP in agar and the two American cultures, *P. chrysosporium* and *P. sordida*, to degrade PCP in aged-residue soil was investigated (Walter et al., 2005b – Chapter 7). Colonised SCS (32% by volume) was mixed with the equivalent of 50 g dry weight of aged residue soil to give a starting concentration of 60 mg/kg PCP. There were two replicate 250 mL specimen containers (Labserv, Biolab) for each isolate. Throughout the experiment the moisture content of the SCS-soil mix was maintained at 83% of container capacity. The percentage of the visible SCS-soil mix colonised (0, 5, 10, 25, 50, 75, 90 and 100%) was visually estimated after 7 days. Degradation was measured after 42 days incubation at room temperature by taking one sample per specimen container and analysing for PCP by HPLC, according to the methods of Walter et al. (2003 – Chapter 2). PCP levels for each isolate after 42 days were compared to a control consisting of an uninoculated SCS-soil mix at day 0.

PCP mineralisation in liquid and soil

Mineralisation studies using ^{14}C-PCP in liquid and soil were conducted as described by Walter et al. (2004 – Chapter 3). Mineralisation of PCP and the presence of pentachloroanisole (PCA), which is a toxic metabolite of PCP, was measured in liquid culture for five New Zealand native white-rot isolates, and for *P. chrysosporium.* In soil microcosm studies, mineralisation of PCP by three New Zealand native white-rot

isolates in soils with a concentration of 50, 200, 1000 and 5000 mg/L PCP was investigated.

Table 9.1. White-rot isolates tested in liquid culture and soil microcosms for PCP degradation

Fungus[a]	Species	Source (other code[b])
HR145	*Abortiporus biemmis*	HortResearch, NZ
HR339	*Australporus tasmanicus*	Forest Research, NZ (FRI 226)
HR345	*Oudemansiella australis*	Forest Research, NZ (FRI 238)
HR226	*Peniophora sacrata*	Forest Research, NZ (FRI 36B)
HR235	*Peniophora sacrata*	Forest Research, NZ (FRI 36K)
HR240	*Peniophora sacrata*	Forest Research, NZ (FRI 36P)
HR241	*Peniophora sacrata*	Forest Research, NZ (FRI 36Q)
HR316	*Rigidoporus catervatus*	Forest Research, NZ (FRI 202)
HR348	*Stereum fasciatum*	Forest Research, NZ (FRI 197)
HR192	*Trametes* sp.	HortResearch, NZ
HR196	*Trametes* sp.	HortResearch, NZ
HR197	*Trametes* sp.	HortResearch, NZ
HR131	*Trametes versicolor*	HortResearch, NZ (Culture A[c])
HR154	*Trametes versicolor*	HortResearch, NZ(Culture B[c])
HR160	*Trametes versicolor*	HortResearch, NZ (Culture C[c])
HR275	*Trametes versicolor*	Forest Research, NZ (FRI 75A)
HR277	*Trametes versicolor*	Forest Research, NZ (FRI 75C)
HR445	*Trametes versicolor*	Landcare Research, NZ (PB86/097a)
HR112	Unknown	HortResearch, NZ
HR122	Unknown	HortResearch, NZ
HR152	Unknown	HortResearch, NZ
HR577	Unknown	HortResearch, NZ
HR589	Unknown	HortResearch, NZ

[a] HortResearch Culture Collection Code

[b] Corresponding Culture Collection Code from supplier

[c] Deposited at Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia with Accession numbers NM02/27875, NM02/27876, and NM02/27877 for Culture A, Culture B, and Culture C, respectively.

Correlation analyses were performed on the three screening tests dye decolourisation, wood decay, and growth on 50 mg/L agar, against PCP degradation in liquid culture (Walter et al., 2003 – Chapter 2).

Field remediation of PCP contaminated soil

Field biopiles with a capacity of 1 m^3 were designed to develop proof-of-concept biopiles for white-rot bioremediation of aged-PCP contaminated soil from a former timber treatment facility. Piles were constructed to allow for forced aeration, irrigation, leachate collection, and monitoring of temperature and soil moisture content. Parameters studied were the effect of a selected white-rot fungus on PCP degradation, the effect of fungal inoculum concentration on PCP degradation and reproducibility of the experiments. PCP degradation and fungal survival were monitored in regular intervals for up to 12 months. Four bins were inoculated with 20% colonised SCS (by volume) and one bin with 40% colonised SCS of HR131. Samples of soils were removed as independent cores from each of the 4 soil cells and analysed for PCP by HPLC. Air temperature and biopile temperatures were recorded with a Campbell Scientific CR10 datalogger (Walter et al., 2005c – Chapter 8).

Results

Growth and survival studies

Temperature studies. There was considerable variation in growth rate, optimum temperature for growth, and tolerance to temperature extremes between genera and species, and within species. Between 0 and 30°C, all isolates grew or resumed growth. Approximately 18% and 40% did not survive incubation at 35 and 40°C respectively (Walter et al., 2003 – Chapter 2).

Ligninolytic activity. For the 367 isolates tested using the polymeric dye assay, 95 isolates (26%), including *P. chrysosporium*, showed obvious discolouration after 7–11 days of incubation. All isolates tested in the lignin degradation assay using willow cuttings caused wood decay ranging from 5 to 169 mm (Walter et al., 2003 – Chapter 2).

Colonisation of New Zealand soil by the white-rot Trametes versicolor. Colonisation of soils ranged from sparse to complete colonisation. Isolate performance was dependent upon soil type. Soil colonisation was affected by base saturation, organic matter content, calcium, phosphorous, and to a lesser extent nitrogen levels (Boyd-Wilson et al., 2005 – Chapter 6).

Growth of selected Trametes versicolor isolates in PCP contaminated soil. Colonisation of PCP contaminated soil differed significantly ($P<0.05$) between the three isolates. Colonisation of all 3 isolates was affected ($P<0.05$) by the presence of PCP in the soil. Colonisation was reduced to 45% in the presence of 100 mg/kg PCP compared to the control treatment (0 mg/kg PCP) (Walter et al., 2005a – Chapter 5).

Laboratory degradation potential studies

Tolerance to PCP in agar. Of the 253 white-rot isolates tested, 38% produced viable growth on 50 mg/L PCP. The two American isolates, *P. chrysosporium* and *P. sordida*, did not grow on 50 mg/L PCP amended agar. Twenty-three of the 95 New Zealand white-rot isolates tolerant to 50 mg/L PCP grew on 200 mg/kg PCP amended agar (Walter et al., 2003 – Chapter 2).

PCP degradation in liquid culture. All 20 isolates tested were found to reduce significantly ($P<0.05$) PCP in the liquid fraction over the 42 day incubation period, when compared to the PCP-control (Figure 9.1). For five of the isolates, no PCP could be detected in the liquid fraction. Ten of the white-rot isolates produced laccase at some stage over the 42 days of the experiment (Walter et al., 2003 – Chapter 2).

Correlation analyses found no relationship between the three screening tests dye decolourisation, wood decay, and growth on 50 mg/L PCP-agar, and PCP degradation *in vitro* (Walter et al., 2003 – Chapter 2).

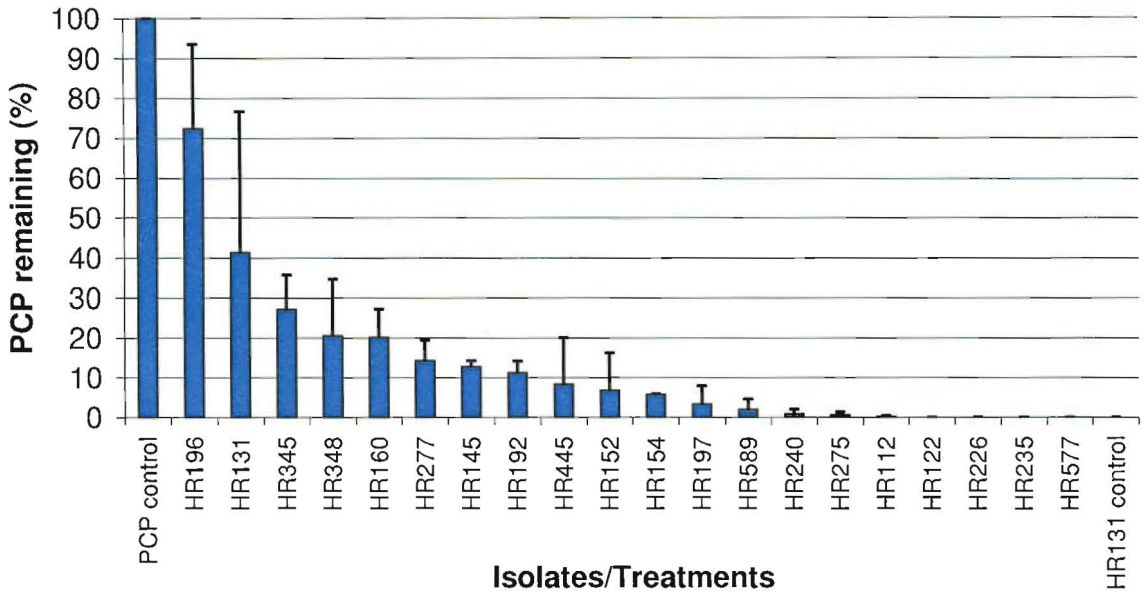

Figure 9.1. Percent of PCP remaining in the liquid fraction after 42 days stationary incubation with white-rot fungi at room temperature. Error bars show the standard deviation of the means (Walter et al., 2003 – Chapter 2, Figure 2.1).

Degradation of PCP in soil microcosms. Growth of fungi in soil microcosms differed significantly ($P<0.001$) between isolates, and ranged from 17.5% colonisation to 100% colonisation of the visible SCS-soil mix. Nine of the isolates tested had degraded PCP significantly ($P<0.01$) after 42 days when compared to the control level at day 0. For isolate HR160, no PCP was detected in the sample after 42 days (Figure 9.2.; Walter et al., 2005b – Chapter 7).

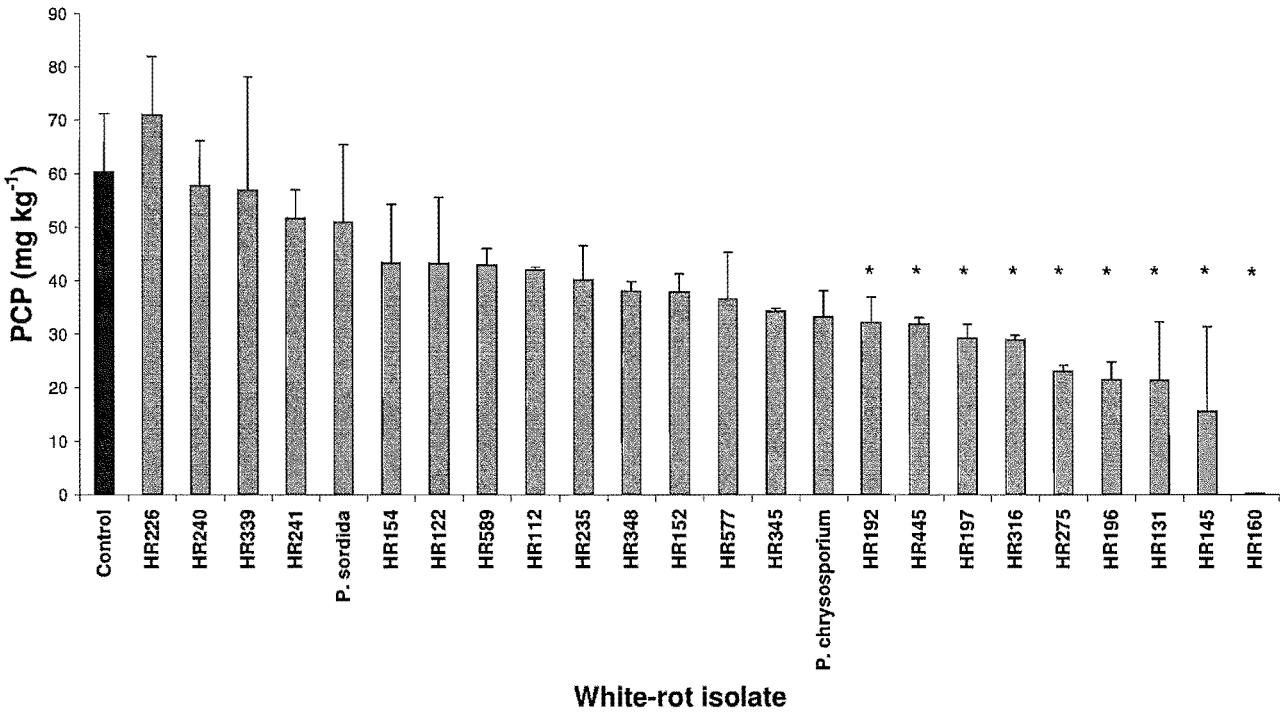

Figure 9.2. PCP remaining in soil after 42 days incubation with white-rot fungi at room temperature. Significant ($P<0.05$) differences from the control are denoted by *. Error bars indicate standard deviation of the means. (Walter et al., 2005b – Chapter 7, Figure 7.2).

PCP mineralisation in liquid and soil. In liquid culture, all five New Zealand white-rot isolates mineralized PCP at a higher rate than *P. chrysosporium* (Figure 9.3). Very little or no PCA was captured for the five native isolates, whereas 75% of the volatile fraction of *P. chrysosporium* consisted of PCA. In the soil microcosms study (Figure 9.4), all three isolates were able to mineralise PCP at concentrations up to 200 mg/L (Walter et al., 2004 – Chapter 3).

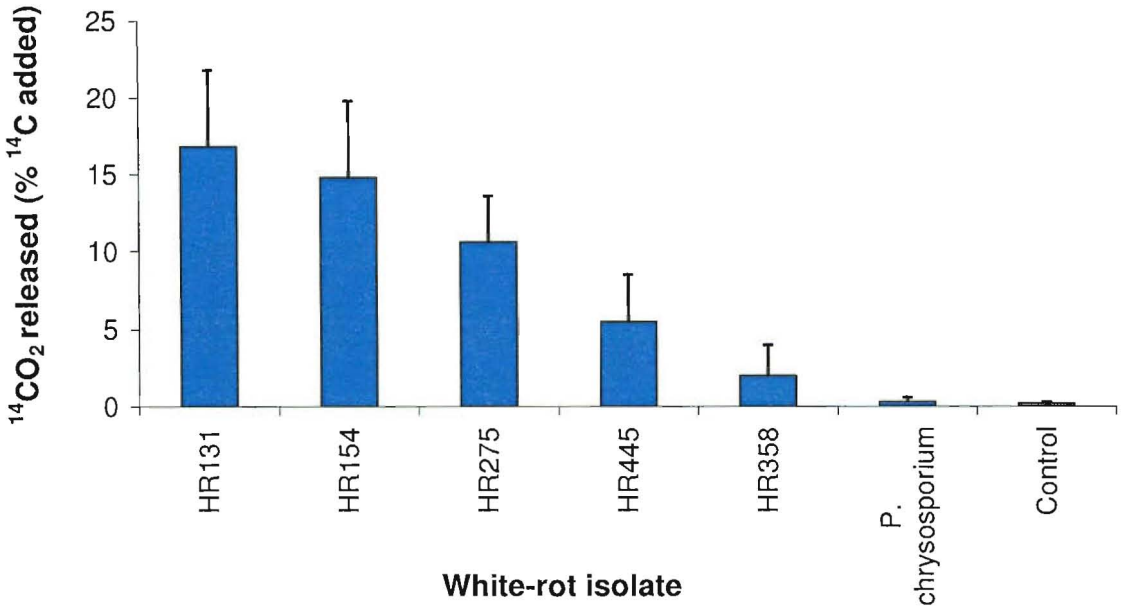

Figure 9.3. Release of $^{14}CO_2$ from 17 kBq ^{14}C-PCP in liquid culture. Error bars indicate standard deviation of the means.

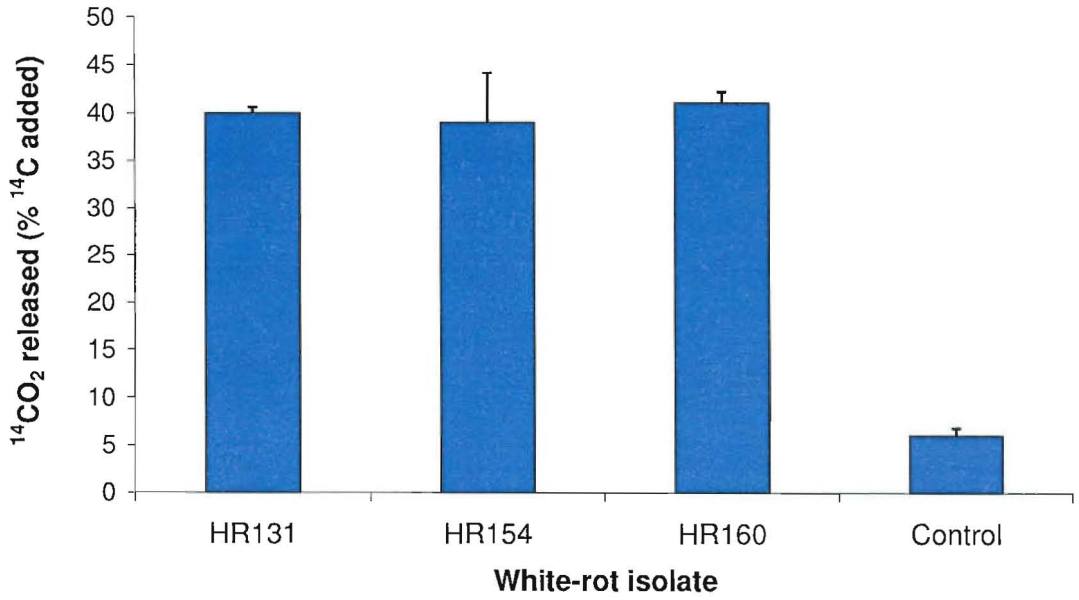

Figure 9.4. Release of $^{14}CO_2$ from ^{14}C-PCP in 200 mg/L aged-PCP soil. Error bars indicate standard deviation of the means.

Field remediation of PCP contaminated soil

Field remediation using HR131 reduced aged PCP residue from approximately 800 mg/kg to 100 mg/kg within one year of treatment and less than 50 mg/kg after 74 weeks (Figure 9.5). Little, or no, PCA was detected during the degradation process. Temperatures inside the biopile remained within 4°C below the air temperature.

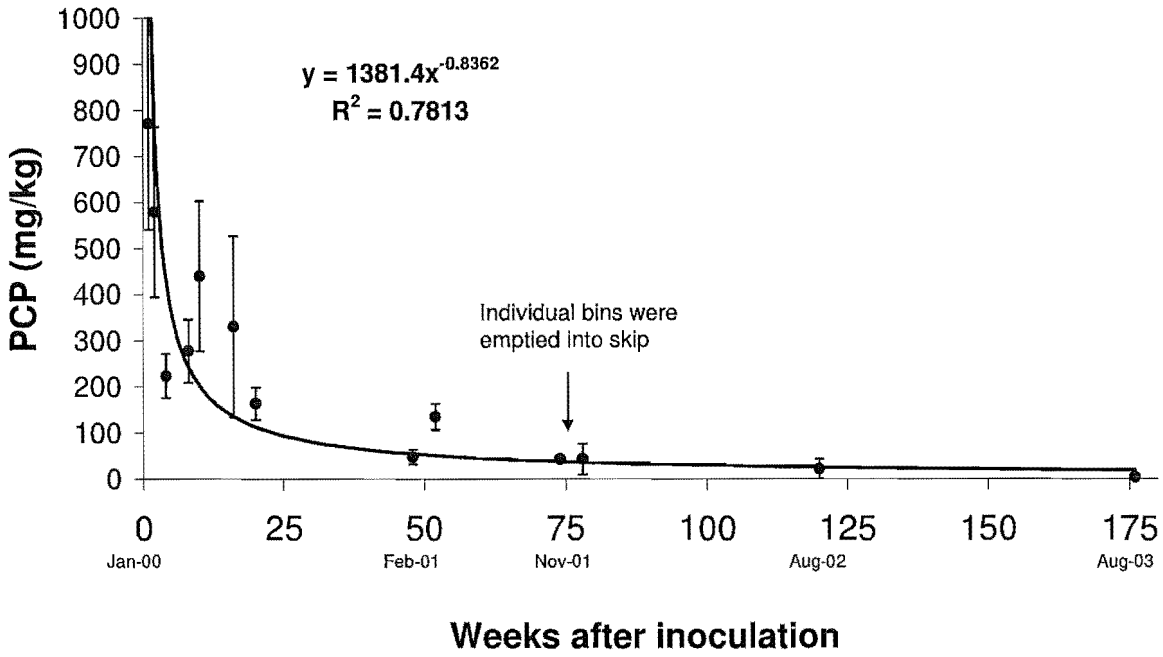

Figure 9.5. Decline in PCP contaminated soil in the field after inoculation with a New Zealand white-rot fungus. Error bars indicate standard deviation of the means (Walter et al., 2005c – Chapter 8, Figure 8.5).

Discusssion and conclusion

Isolate specific effects highlight the importance of a detailed database of growth and survival characteristics under specific conditions in order to select isolates for transfer to the field.

All isolates survived temperatures of between 0 and 30°C. As temperatures within the biopiles stayed within this range, biopile temperature would not have limited growth of these isolates.

All isolates showed ligninolytic activity by decaying willow cuttings. The polymeric dye indicating hydrogen peroxidase activity and the laccase assay identified

isolates that produced certain lignin-modifying enzymes. Research into ligninolytic enzymes and pathways of degradation by *Trametes versicolor* for PCP degradation continues as a complementary project.

Certain isolates were highly tolerant to PCP in agar, as compared to the findings of Alleman *et al.* (1992) who reported that a PCP concentration of 5 mg/L stopped growth of all six species studied. Although tolerance did not correlate with PCP degradation *in vitro*, increased tolerance to the pollutant may benefit fungal survival in soil upon augmentation into the polluted soil environment.

Degradation studies in liquid culture and soil microcosms identified isolates capable of degrading PCP *in vitro*. In addition, five New Zealand native white-rot isolates produced little to no PCA compared to the American isolate of *P. chrysosporium* where PCA accumulated in the volatile fraction. PCA is more toxic than PCP, therefore production and accumulation of PCA could pose an environmental risk. Research to date showed that decline in PCP concentrations *in vitro* was due to mineralisation and biodegradation activities.

The lack of relationship between screening tests and PCP degradation liquid culture emphasises the importance of screening isolates in soil microcosms before selection for transfer to the field.

Proof-of-concept biopiles in the field further demonstrated New Zealand a native white-rot isolates capable of degrading PCP from 800 mg/kg to less than 50 mg/kg in 74 weeks. Prolonged incubation for 2 more years further reduced the PCP levels to less than 5 mg/kg (Walter et al., 2005c – Chapter 8). The field research and the prototype soil cells also advanced our current understanding of the engineering requirements for successful field remediation using native white-rot fungi.

Acknowlegements

We would like to thank the Foundation for Research, Science and Technology for funding. Thanks to all colleagues and staff involved in the research to date. Thanks to Forest Research, Landcare Research, AgResearch, WRONZ, URS (formerly Woodward-Clyde), Utah State University (USA), Massey University and University of Canterbury for isolates and/or research collaboration. Special thanks to Environment Canterbury for providing the field site and to all regional/local councils and industry partners for their support and interest in the project.

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Chapter 10

General Discussion

Aims of thesis

The aims of this thesis were to evaluate selected white-rot fungi for their ability to decontaminate soils by degradation of PCP in the field by:

4) quantifying PCP loss and breakdown associated with each fungal isolate,
5) identifying limitations (e.g. isolate, soil type, moisture, temperature, pollutant concentration) and
6) optimising fungal activity for efficient breakdown of PCP.

To achieve these goals, process monitoring tools were required for tracking survival and activity of white-rot fungi in the biopile as well as the development of microcosm studies and treatability analyses to determine the soil factors influencing white-rot remediation and predict pollutant degradation by selected isolates in the field, respectively.

The research programme, described in this thesis, achieved its aims and signifies not only an important step in New Zealand towards white-rot fungal bioremediation, but also internationally towards bioremediation of PCP using *T. versicolor*.

Summary of bioremediation research

Generally, bioremediation research can be grouped into seven or more interrelated program elements.

1. Biotransformation and biodegradation

Further knowledge is required of mechanisms and pathways for biotransformation and biodegradation for single pollutants and mixtures of contaminants, including metals. This is mostly research discovery area, with some potential for intellectual

property relating to identifying or creating superior enzymes or triggers for superior enzyme activity.

2. *Community dynamics and microbial ecology*

Knowledge is required for understanding the composition, structure and function of organisms present in natural systems and their interaction with, and response to, pollutants and remediation treatments. The role of environmental factors influencing microbial consortia and how these factors and organisms (including white-rot fungi) work together to degrade or transform contaminants should be determined.

3. *Biomolecular science and engineering*

The potential of molecular manuipulation to enhance bioremediation needs to be determined. To realize the potential, information is required to analyse genes, proteins and regulatory elements of critical molecules for bioremediation. Knowledge of structure and function relationships is also needed to understand the enzymatic mechanisms for detoxification. Building on this information, organisms can be specifically selected from breeding programmes/culture collections for certain gene-expression and/or engineered with superior bioremediation abilities.

4. *Biogeochemical dynamics*

Biogeochemical dynamics involves method development for measuring the *in situ* distribution of organisms with potential for biodegradation and for understanding the environmental factors that control these distributions. These are particularly required for natural attenuation/accelerated natural attenuation using stimulants. Also included in this category is understanding the biogeochemical processes and how they interface and are affected by nature, e.g. role of solid-liquid interfaces, liquid-gas interfaces, interface between immiscible liquids and the bioavailability of contaminants and nutrients.

5. *Assessment*

Real-time measurement is needed to monitor the processes responsible for bioremediation and assessing their efficacy. This includes methods for assessing biodegradation rates and biodegradation activities (e.g. fungal survival and mycelial extension, enzyme activity). This requires development of non-invasive methods or minimally invasive techniques for site characterisation, monitoring loss of

contaminants/biodegradation rate, diagnostic techniques for interpretation of measurements, and developing a scientifically sound strategy for identifying achievable bioremediation end-points.

6. Acceleration

The factors that limit the rate of bioremediation in the field need to be better understood in order to develop methods for the acceleration of biogeochemical processes/fungal processes. This may include increasing bioavailability of contaminants, supplying nutrients or fertilisation, exploiting the underlying transport processes, developing innovative biostimulation/bioaugmentation methods as well as developing new inoculation techniques for fungal inocula into polluted sites and/or formulation approaches such as liquid formula, pellets, the use of bulking substrates and co-substrates that increase whit-rot fungal activity in polluted soils.

7. System integration, prediction and optimisation

Knowledge is required to predict and optimise biodegradation processes and community dynamics with the aid of mechanistic models that quantitatively describe biotransformation/biodegradation processes, community dynamics and biogeochemical interfaces. This will integrate the knowledge above on assessment, acceleration, biogeochemical processes, community dynamics and biotransformation, and will become instrumental in engineering designs for remediation approaches at field scale, as well as information dissemination.

8. Social issues

Impact assessment is needed on measuring the effect of pollution versus remediation (or degree of remediation) on the environment, on the local industry and on the society/country. Impact assessment of different remediation approaches on the environment (e.g. does solving one problem create a new one?) including cost-benefit analysis. This should be linked in to legal and social issues around polluted soil sites, as well as ownership and liability issues.

While other groupings are possible (e.g. according to science disciplines; pollutant type; biodegrading organism; and contaminated substances such as water, soil and air), applied bioremediation requires an interdisciplinary approach between the various

program elements/science disciplines. This is also reflected in this PhD research where a range of science disciplines (e.g. mycology, fungal biology, enzymology, microbial ecology, soil science, chemistry (including analytical chemistry), molecular biology, formulation technology and engineering) were integrated into the project. The overlap of the science disciplines is also reflected in the co-authorship of publications submitted/to be submitted due to the expert-assistance provided by outside advisers to the student.

The applied nature of the work is also reflected in this thesis, since the research was process-oriented rather than science-driven to facilitate commercial application of *T. versicolor* for bioremediation of PCP polluted sites in New Zealand. The research presented was therefore somewhat delimited by the 'commercial focus'. For example, the use of radiolabelled PCP would have added valuable information on pollutant degradation in the laboratory based treatability tests. However, the aim was to develop systems and protocols suitable for commercial treatability studies. Waste management companies usually do not have access to the special facilities and instruments required for working with radiolablelled pollutants. A parallel approach (using both non-labelled and ^{14}C-labelled PCP) was beyond the resources of the student.

Future research needs

Some of the fundamental aspects touched in the thesis require more investigative science, such as pollutant aging processes; binding of pollutants and metabolites to soil/soil matter; bioavailability of aged pollutants; indigenous soil microbial-white-rot fungus-pollutant interactions; inducers of pollutant degrading enzymes in soil; fungal genetics – to name just a few. The list of fundamental research needs is vast and follows the groupings explained above. However, despite these fundamental knowledge gaps, the applied proof-of-concept on 'PCP degradation by New Zealand native white-rot fungi' was successful. From the base established by this PhD research, follow-on applied and fundamental research could include the following studies.

1. Biotransformation and biodegradation

- Biochemical mechanisms (e.g. enzymatic pathways) involved in *T. versicolor* degradation of pollutants.
- Pathways of pollutant degradation – catabolic and metabolic processes.

- Fate of pollutant and degradation products in soil environments augmented with white-rot fungi, including adsorption and humification processes.
- White-rot fungi and their role as biological catalysts.
- Analytical technologies - the limitations of chemistry create a need for biosensors.

2. *Community dynamics and microbial ecology*

- Structure of microbial communities and their dynamics in response to presence of white-rot fungi and their dynamics in biodegradation – microbial ecology, physiology and genetics.
- Single isolate treatment technologies versus mixed populations: how do different strains, species or genera interact in bioremediation processes?
- The fungal highway: mycelial cords and oxygen/nutrient transport in bioremediation.

3. *Biomolecular science and engineering*

- Microbial genetics for enhancing the capability of white-rot fungal organisms to degrade pollutants.
- The superbug: producing the superbug via genetical engineering or protoplast fusion?
- White-rot fungi genetics and pollution degradation.

4. *Biogeochemical dynamics*

- Bioavailability and biodegradation: how to access low concentrations of contaminants.

5. *Assessment*

- Develop and evaluate biotechnologies for monitoring bioremediation in the field.
- Develop reliable and uniform methods for assessing efficacy of white-rot bioremediation technologies.
- Ecotoxicology: the impact of break-down products.
- Microbial indicators versus analytical chemistry.
- Ageing – the chemical, microbial and physical processes in the presence and absence of white-rot fungi.

- Extraction efficacy of bound residues and bioavailability of aged residues in the presence and absence of white-rot fungi.

6. *Acceleration*

- Accelerated fungal bioremediation by manipulating the soil environment and overcoming soil-specific limitations (aeration, surfactants, nutrient balances).
- Fungal inoculum production: 'ready to use product'.
- White-rot biotechnology: whole organisms versus enzyme applications for bioremediation.

7. *System integration, prediction and optimisation*

- Modelling of biological processes at work in bioremediation.
- Biopile design – landfarming versus engineered soil cells.
- Which remediation system should be used? Life cycle assessments for balancing cost, remediation efficacy and other environmental and social impacts. Are there situations when white-rot treatments should not be used?
- Special pollutants: e.g. POP's (dieldrin, dioxins) and PAHs. How transferable is the PCP-model system?
- When to treat and when not to treat – remediation versus natural attenuation.

8. *Social issues*

- Centralised treatment facilities or mobile units?
- Improved engineering for white-rot biopiles, eg integration with ecological engineering?
- Application for pollution prevention – bioreactors for water/air treatment?
- How safe are bioremediation technologies?
- Are there side effects to white-rot treatment technologies?
- Are white-rot isolates used in bioremediation applications pathogenic to native and exotic tree species?

The research programme described in this thesis, provides a suitable platform to further explore the future research needs.

Chapter 11

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Appendices

Appendix Chemical Residue Analysis

For

Chapter 2

Chapter 3

Chapter 7

Chapter 8

Chapter 9

Chemical Residue Analysis

for

Chapter 7

Chapter 9

Conducted by HortResearch, Ruakura Research Centre, Hamilton

Contact: Grant Northcott

PCP extraction and analysis

Approximately 5 grams of homogenous solid sample (wet weight as received) and 2 ml of concentrated phosphoric acid were added to a glass bottle and shaken by hand to mix. 100 ml of acetone/hexane (2:3 v/v) was added and shaken by hand to mix before extraction by sonication for 10 minutes (50 kHz, Kerry Pulsatronic KS400 unit; Kerry, Hertfordshire, United Kingdom) and mechanical shaking for 60 minutes on a flat bed shaker (IKA KS 500, 300 cycles/minute). After shaking, 150 ml of ultrapure water (Millipore, Milford, MA, USA) was added to separate the hexane phase containing extracted PCP. An aliquot of the hexane solution was transferred to a 2 ml glass HPLC vial and 0.02 ml of iso-butanol added as keeper solvent. Hexane was removed under a gentle stream of nitrogen gas at room temperature and 1.6 ml methanol/water (80:20 v/v) added immediately to the vial, which was capped. The contents of the vial were mixed by vortex and stored under refrigeration until analysis by HPLC.

Liquid chromatographic separation and detection of PCP were performed using a Shimadzu LC-10A liquid chromatography system and Shimadzu SPD-10AV UV-vis detector at 280 nm. Chromatography was performed at 35°C with an isocratic methanol/water/acetic acid mobile phase (81/18/1% by volume) and 5 µm, 150 x 4.5-mm-i.d.Luna C_8 reversed phase column (Phenomenex NZ Ltd). The column flow rate was 1.0 ml/min and injection volume 10 µl. Six calibration standards (50, 20, 10, 5, 2.5, 1 µg/ml) were analysed with each batch of samples and PCP in the samples was quantified against the constructed calibration curve. PCP recovery during sample extraction and workup was determined for each batch of extracted samples (maximum of twenty) by spiking solvent blanks and control soil samples with a known amount of PCP. PCP recovery was typically 90 to 100% and the amount of PCP measured in a corresponding batch of samples was corrected by the appropriate recovery factor for spiked samples and the blank value.

Note: this method has been published by

Monika Walter[1], Kirsty S.H. Boyd-Wilson[1], Don McNaughton[2] and Grant Northcott[2]*

"Laboratory trials on the bioremediation of aged pentachlorophenol residues." In International Biodeterioration and Biodegradation (2005) Volume 55, Page 121-130.

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Chemical Residue Analysis

for

Chapter 3

Chapter 9

Conducted by LincLab, Canesis Ltd, Lincoln

Contact: Bob Foulkes

^{14}C Labelled PCP in Soils

The soil samples provided were mixed and processed according to the method provided. To ensure the total non-volatile ^{14}C labelled PCP was taken into account for a final mass balance check on the extraction, the method was modified to retain and accumulate all rinsings etc for counting.
Thus the method becomes:

Materials:

1. Extraction solution: 50/50 ACO/HEX, saturated with 2.5M HCl
2. Wash solution: 50/50 0.2M HCl / saturated NaCl
3. Rinse Solution: 50/50 DCM/HEX

Method:

1. sub-sample soil and determine moisture content separately.
2. extract 10g fresh weigh of soil with 20ml extraction solution in a 50ml Teflon centrifuge tube by end over end shaking for 1 hour. (As the shaker broke during this work this step was replaced by ultrasonication for 30min for some samples.)
3. centrifuge and transfer supernatant to a separating funnel containing 20ml of wash solution.
4. re-extract the disrupted soil pellet with 10ml extraction solution shaking for 10 min.
5. centrifuge and transfer the supernatant to the separating funnel.
6. repeat 4-5.
7. shake the funnel for 30 seconds to wash the hexane layer, and when the phases have separated run off the aqueous acetone wash solution into a 100ml collection bottle. This is sample C.
8. repeat 7, accumulating the washings.
9. collect the spent extracted soil, transferring it with water into a sealable container and allow to air dry. This sample can then be combusted for residual unextracted ^{14}C labelled material. This is sample E.

10. pass the washed hexane extract from step 7-8 above through a 500mg silica SPE cartridge hand packed full with anhydrous Na_2SO_4 (approx 2g). Adjust the flow to around 2ml/min, collecting the eluate into 50ml volumetric flasks. Rinse the funnel with 5ml hexane.
11. After the hexane extract has passed through the SPE rinse the reservoir and the SPE with 5ml Rinse Solution, collecting all rinsings into the volumetric flasks.
12. Remove the spent SPE cartridge and rinse with 10ml Acetone, collecting the rinsings into a separate collection bottle. This is sample D
13. make the eluates from step 11 up to 50ml and transfer duplicate 2.5ml portions to scintillation vials containing counting cocktail and cap tightly. These are samples B1 and B2.
14. warm the volumetric flasks on a hot plate with a nitrogen purge to concentrate the remainder of the collected eluate down to ≈5ml, then transfer quantitatively to concentrator flasks. Dry the residue at 60°C to near dryness, then redissolve into 1ml THF and transfer to GC vials and cap with Teflon septa. This is sample A

Thus the 14C labelled PCP has been divided into the following sub-samples:

A. 1ml concentrated THF solutions supposedly containing 95% of the original labelled material extracted from the soil. These have been analysed by GCMS and recapped. GCMS consumes around 50µl of sample i.e. ≡ 5% of total of 1 ml.
B. Duplicate samples in cocktail ready for counting each supposedly containing 2.5% of the original labelled material extracted from the soil.
C. 5ml sub-sample from ≈40ml of the aqueous washings, supposedly containing none of the original labelled material extracted from the soil.
D. 10ml acetone extract of the SPE in cocktail ready for counting, supposedly containing none of the original labelled material extracted from the soil.
E. Near quantitative residues of the air dried soil after extraction, supposedly containing none of the original labelled material present in the soil.
F. This additional sample is oven-dried unextracted soil, weighed and used for moisture determination.

Scintillation Counting / Chromatography:

Originally the samples were to be analysed by fractionation on RP-HPLC followed by fraction counting with an off line scintillation counter. However an on-line HPLC scintillation detector was available at Lincoln Ventures, and this offered the possibility of not only counting the labelled material but speciating it as well. Unfortunately the ratio of labelled PCP to unlabelled material was very low. When sufficient sample was injected to ensure a detectable activity of labelled PCP, the unlabelled material massively overloaded the column, greatly distorting the peaks and making the technique unusable.

Off-Line Scintillation Counting of Fractions:

1. Inject 200µl (≅20%) of the sample from step 14 above into the HPLC and collect 1ml fractions into 1.5ml centrifuge tubes. Quantitatively transfer these fractions to vials with cocktail and count.
2. Transfer 2 duplicate 200µl (≅20% each) samples into scintillation vials with cocktail and count.
3. Quantitatively transfer the rest of the sample (≅550µl) into a further vial with cocktail and count. The volume of this sample should be (≅600µl) but a portion of this sample (50µl ≅5%) has already been removed for analysis by scintillation HPLC.

The total of the counts from steps 1-3 thus represents ≅95% X 95% ≅ 90.25% of the labelled material extracted by the extraction process.

Bob Foulkes / 30/03/2000

Chemical Residue Analysis

for

Chapter 2

Chapter 3

Chapter 8

Chapter 9

Conducted by LincLab, Canesis Ltd, Lincoln

Contact: Bob Foulkes

PCP in Soils

By WRONZ for Monika Walter's PhD studies

The moist soil samples provided were mixed and processed according to the following method.

Materials:

4. Extraction solution: 50/50 ACO/HEX, saturated with 2.5M HCl
5. Wash solution: 50/50 0.2M HCl / saturated NaCl
6. Rinse Solution: 50/50 DCM/HEX

Method:

15. Sieve the total soil sample through 2mm mesh, collecting all the soil that freely passes through. For most samples around 25% passes. Return the reject soil to the original container. Mix the sieved soil sample thoroughly by shaking in an inflated snap-lok bag. Sub-sample and determine the moisture content at the same time, weighing around 10g sample before and after drying at 105°C for 24hrs.
16. Extract 10.0±0.1g fresh weigh of soil with 20ml extraction solution in a 50ml Teflon centrifuge tube by end over end shaking for 1 hour. (As the shaker broke during this work this step was replaced by ultrasonication for 30min for some samples.)
17. Centrifuge and transfer supernatant to a 100ml separating funnel containing 20ml of wash solution.
18. Re-extract the disrupted soil pellet with 10ml extraction solution shaking for 10 min.
19. Centrifuge and transfer the supernatant to the separating funnel.
20. Repeat 4-5.
21. Shake the funnel for 30 seconds to wash the hexane layer, and when the phases have separated run off the aqueous wash solution to waste.
22. Repeat 7.
23. Pass the washed hexane extract from step 7-8 above through an ALLTECH 500mg Maxiclean silica SPE cartridge hand packed full with anhydrous Na_2SO_4 (approx

2g). Adjust the flow to around 2ml/min, collecting the eluate into 50ml volumetric flasks. Rinse the funnel with 5ml hexane.

24. After the hexane extract has passed through the SPE rinse the reservoir and the SPE with 5ml Rinse Solution, collecting all rinsings into the volumetric flasks, and make up to 50ml with hexane. Transfer 2 aliquots of approximately 1ml each to GC vials and cap with Teflon septa ready for GCMS SIM analysis (PCPSIM3.MET).

Bob Foulkes / 30/03/2000

Appendix to Chapter 2

PCP residue data

isolate number	Lincoln I.D. number	poster isolate no.	rep no.	ug PCP/ml HPLC	ave ug PCP/ml HPLC	stdev	CV	% PCP of control
HR112	enz1F1	1	1	0	0.015	0.021213	141.4214	0.15
HR112	enz1F2	1	2	0.03				
HR122	enz1F3	2	1	0	0	0	0	0
HR122	enz1F4	2	2	0				
HR131	enz1F5	6	1	6.46	4.03	3.436539	85.27392	41.35
HR131	enz1F6	6	2	1.6				
HR145	enz1F7	7	1	1.14	1.245	0.148492	11.9271	12.78
HR145	enz1F8	7	2	1.35				
HR152	enz1F9	8	1	1.31	0.655	0.92631	141.4214	6.72
HR152	enz1F10	8	2	0				
HR154	enz1F11	9	1	0.55	0.56	0.014142	2.525381	5.75
HR154	enz1F12	9	2	0.57				
HR160	enz1F13	10	1	2.45	1.96	0.692965	35.35534	20.11
HR160	enz1F14	10	2	1.47				
HR192	enz1F15	11	1	1.3	1.095	0.289914	26.47614	11.24
HR192	enz1F16	11	2	0.89				
HR196	enz1F17	12	1	5.58	7.045	2.071823	29.40842	72.29
HR196	enz1F18	12	2	8.51				
HR197	enz1F19	13	1	0.64	0.32	0.452548	141.4214	3.28
HR197	enz1F20	13	2	0				
HR226	enz1F21	14	1	0	0	0	0	0
HR226	enz1F22	14	2	0				
HR235	enz1F23	15	1	0	0	0	0	0
HR235	enz1F24	15	2	0				
HR240	enz1F25	4	1	0.16	0.08	0.113137	141.4214	0.82
HR240	enz1F26	4	2	0				
HR275	enz1F27	3	1	0.11	0.055	0.077782	141.4214	0.56

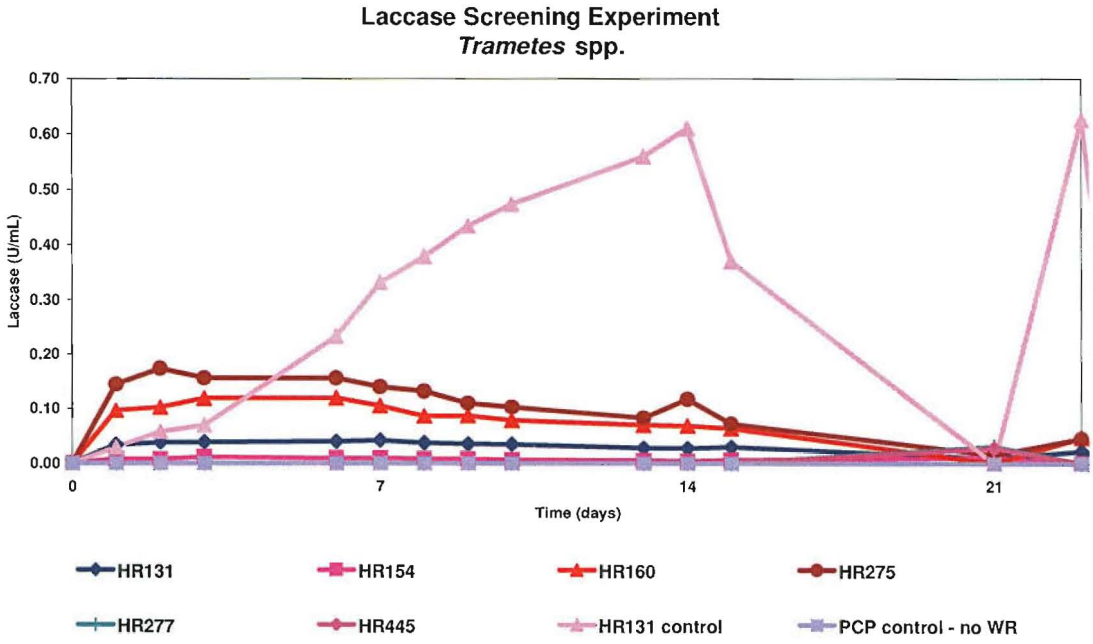
HR275	enz1F28	3	2	0				
HR277	enz1F29	16	1	1.039	1.395214	0.503195	36.06577	14.32
HR277	enz1F30	16	2	1.751				
HR345	enz1F31	17	1	2.066	2.650808	0.827247	31.20736	27.20
HR345	enz1F32	17	2	3.236				
HR348	enz1F33	18	1	1.019	1.998644	1.385506	69.32228	20.51
HR348	enz1F34	18	2	2.978				
HR445	enz1F35	19	1	1.620	0.81	1.145512	141.4214	8.31
HR445	enz1F36	19	2	0.000				
HR577	enz1F37	5	1	0	0	0	0	0
HR577	enz1F38	5	2	0				
HR589	enz1F39	20	1	0.369	0.184614	0.261083	141.4214	1.89
HR589	enz1F40	20	2	0.000				
HR131 control	enz1F41	21	1	0	0	0	0	0
HR131 control	enz1F42	21	2	0				
PCP control	enz1F43	control	1	15.91	9.745	8.718627	89.46769	100
PCP control	enz1F44	control	2	3.58				

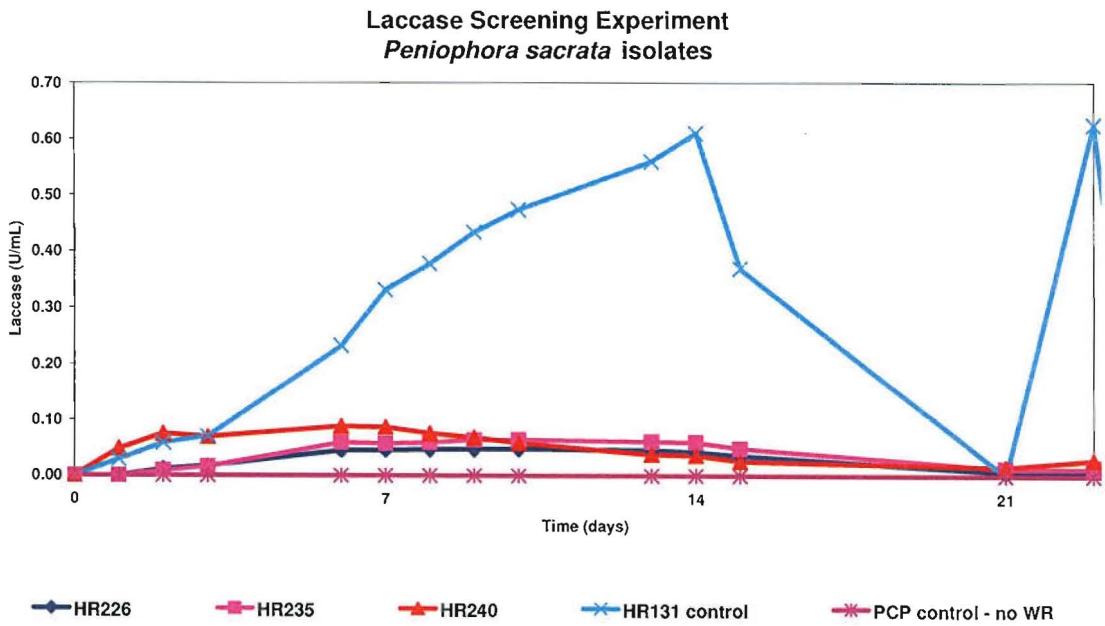
Laccase data (U/mL)

Flask no.	WR code	rep	Slope D1	Slope D2	Slope D3	Slope D6	Slope D7	Slope D8	Slope D9	Slope D10	Slope D13	Slope D14	Slope D15	Slope D21	Slope D23
1	HR112	1	0.0273	0.0448	0.0447	0.0245	0.0215	0.0165	0.0129	0.0124	0.0164	0.0067			0.0023
2	HR112	2	0.0243	0.0263	0.0294	0.0138	0.0148	0.0112	0.0096	0.0081	0.0106	0.0071	0.0072	0.0385	0.0434
3	HR122	1	0.0174	0.0194	0.0228	0.0206	0.0214	0.0172	0.0161	0.0160	0.0254	0.0201	0.0191	0.0153	0.0060
4	HR122	2	0.0187	0.0229	0.0255	0.0207	0.0211	0.0173	0.0162	0.0144	0.0241	0.0199	0.0189		0.0000
5	HR131	1	0.0370	0.0367	0.0378	0.0387	0.0394	0.0335	0.0314	0.0323	0.0260	0.0261	0.0280	0.0245	0.0231
6	HR131	2	0.0199	0.0282	0.0281	0.0296	0.0323	0.0298	0.0282	0.0256	0.0216	0.0202	0.0223	0.0174	0.0152
7	HR145	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000			
8	HR145	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000			
9	HR152	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
10	HR152	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
11	HR154	1	0.0077	0.0071	0.0102	0.0077	0.0081	0.0064	0.0068	0.0054	0.0067	0.0025	0.0041		0.0000
12	HR154	2	0.0047	0.0049	0.0088	0.0069	0.0084	0.0065	0.0057	0.0052	0.0087	0.0071	0.0054		0.0012
13	HR160	1	0.0971	0.1145	0.1264	0.1290	0.1107	0.0904	0.0929	0.0863	0.0772	0.0809	0.0739	0.0551	0.0592
14	HR160	2	0.0678	0.0603	0.0772	0.0759	0.0694	0.0570	0.0561	0.0484	0.0424	0.0361	0.0346	0.0359	0.0209
15	HR192	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
16	HR192	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
17	HR196	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
18	HR196	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
19	HR197	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
20	HR197	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
21	HR226	1	0.0000	0.0107	0.0148	0.0400	0.0395	0.0390	0.0404	0.0396	0.0342	0.0297	0.0254	0.0103	0.0086
22	HR226	2	0.0000	0.0091	0.0139	0.0363	0.0376	0.0399	0.0391	0.0410	0.0427	0.0413	0.0351	0.0118	0.0063
23	HR235	1	0.0000	0.0071	0.0142	0.0507	0.0498	0.0550	0.0649	0.0661	0.0694	0.0703	0.0529	0.0150	0.0127
24	HR235	2	0.0000	0.0086	0.0127	0.0487	0.0469	0.0447	0.0439	0.0431	0.0330	0.0306	0.0293	0.0103	0.0092
25	HR240	1	0.0419	0.0625	0.0568	0.0743	0.0667	0.0610	0.0606	0.0542	0.0514	0.0528	0.0366	0.0414	0.0318
26	HR240	2	0.0401	0.0640	0.0606	0.0748	0.0798	0.0666	0.0546	0.0443	0.0137	0.0095	0.0074		0.0166
27	HR275	1	0.1258	0.1540	0.1384	0.1371	0.1208	0.1161	0.0964	0.0898	0.0737	0.0717	0.0645	0.0476	0.0420
28	HR275	2	0.1205	0.1411	0.1278	0.1290	0.1181	0.1095	0.0916	0.0860	0.0684	0.1288	0.0583	0.0376	0.0354
29	HR277	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
30	HR277	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
31	HR345	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
32	HR345	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
33	HR348	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
34	HR348	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
35	HR445	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
36	HR445	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
37	HR577	1	0.0000	0.0073	0.0073	0.0070	0.0063	0.0056	0.0051	0.0054	0.0063	0.0062	0.0056		0.0022
38	HR577	2	0.0000	0.0102	0.0103	0.0086	0.0093	0.0064	0.0070	0.0062	0.0082	0.0081			0.0020
39	HR589	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000				
40	HR589	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000						
41	HR131	1	0.0273	0.0525	0.0670	0.2095	0.2592	0.3382	0.3576	0.4087	0.4449	0.5293	0.0790		0.0620

42 HR131 PCP	2	0.0217	0.0453	0.0516	0.1864	0.3042	0.3042	0.3797	0.3966	0.5063	0.5077	0.5481	0.0443
43 control PCP	1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000					
44 control	2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000					

Laccase data presented graphically for selected isolates:





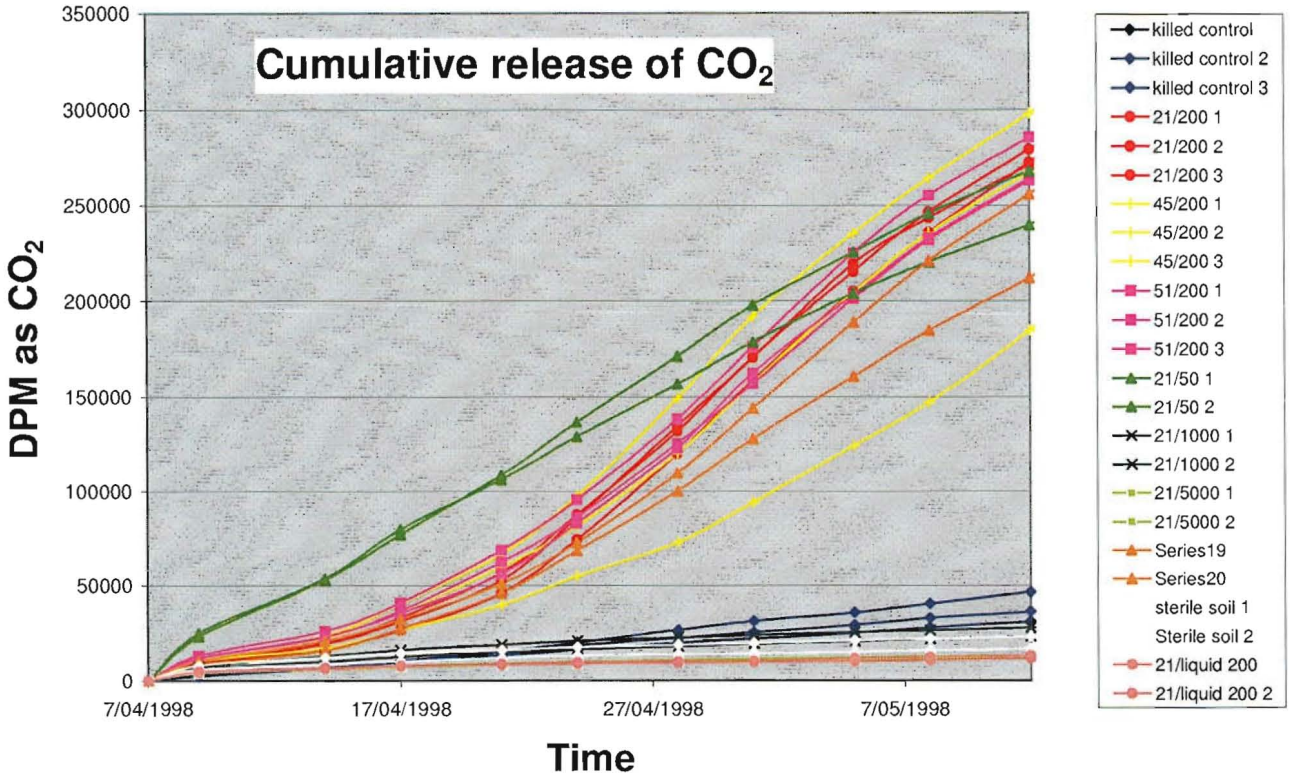
Appendix to Chapter 3

PCP residue data

Soil experiment 1: $^{14}CO_2$ release

Bottle	Treatment	Fungus killed	PCP	7/04/1998	9/04/1998	14/04/1998	17/04/1998	21/04/1998	24/04/1998	28/04/1998	1/05/1998	5/05/1998	8/05/1998	12/05/1998	15/05/1998
1	1	HR131	200	0	2460.4	6671.5	10289.7	13451.7	16418.6	19862.7	22122.1	25149.2	27966.2	31187.6	33341.7
2	1	HR131 killed	200	0	2894.2	6563.1	9765.1	15223.4	20080.7	26632.5	31449	35907.5	40689.8	46826.4	51214.3
3	1	HR131 killed	200	0	2686.7	6693	10356.5	15656.5	18749.9	22558.6	25364	29290.3	32999.4	36331.4	39471.4
4	2	HR131	200	0	8774	19304	31135.8	53052.8	86127.1	134678.8	170608.4	219380.2	243313.1	271245.9	291037.7
5	2	HR131	200	0	8364.9	17156.7	26561.3	46285.6	74704.7	120043.7	158566.3	204986	235648.5	272557.9	299237.6
6	2	HR131	200	0	9068.3	19838	33302.1	56894.7	87712.8	131911.4	171005.6	215422.9	246422.8	279363.2	301853.2
7	3	HR154	200	0	10026.9	17254	26862.9	40203	55233.3	73191.2	94412.5	124291.5	147208.7	184844.8	218695.8
8	3	HR154	200	0	10988.8	22422.3	36406.9	60162	81868.7	120254.2	157545.8	204541.9	235364.4	267535.6	289199.3
9	3	HR154	200	0	11237.3	23702.9	40675	67042.2	97653.1	149279.6	192145.2	235338.3	264399.9	298086.3	326585.7
10	4	HR160	200	0	11581.4	22809.5	36785.8	62686.8	86307.6	125460.9	156936.8	201238.3	231735.6	263189.7	287406.7
11	4	HR160	200	0	11653.9	23118.9	35361.3	56282.5	83402.9	123023.9	162526.9	203017.4	232671.7	264387.9	289167.9
12	4	HR160	200	0	13123.8	26043.7	41027.1	69108.9	95537.2	138363.5	176221.3	224938.5	255411.1	285693.6	308242.2
13	5	HR131	50	0	25084.4	54029.8	80144.4	106388.8	129042.6	156782.8	178795.7	204131.5	220366.3	239814.6	255852.1
14	5	HR131	50	0	23386.3	53401.6	77534.5	108711.8	136625.8	171458.3	197845	225353.6	245846.2	268127.8	283057.1
15	6	HR131	1000	0	7268.5	12793.8	16142.9	18911.3	20865.4	22749.2	24005.5	25560.5	26598.8	28005.3	29147.9
16	6	HR131	1000	0	5997.6	9886.8	12457	14980.5	16542.7	18178.3	19497.9	20823.8	22025.2	23328.8	24329.9
17	7	HR131	5000	0	4196.1	6722	7832.2	9141.3	9952.4	10804.8	11491	12240.8	12665.4	13200.5	13577.4
18	7	HR131	5000	0	3917	6156.6	7375.4	8435.5	9172	9920	10453.2	10998.6	11380.5	11864.5	12283.8
19	8	HR131	200	0	8119.9	15899.4	27494.1	46303.8	68961.9	100235.9	127998.5	160817.7	184717.9	212153.5	237858.8
20	8	HR131	200	0	10208.1	20863.6	32374.4	51316.8	73112.6	109743.5	144030.5	188973.3	220979.2	256001.5	283305.7
21	9	HR131	200	0	7923.3	12142.2	14540.9	16163.8	17657.8	18896	19813.9	21162.4	22172.7	23218.2	24055.8
22	9	HR131	200	0	5873.8	8536.6	10178.5	11982.7	13134.8	13966.5	14480.5	15194.4	15931.6	16790.9	17532.5
23	10	HR131	200	0	4286.7	6207.4	7542.3	8668.2	9452.7	9919.8	10205.1	11567.3	12413.4	13223.3	13922.2
24	10	HR131	200	0	4604.3	6574.1	7701	8431.8	8966.4	9410.9	9716.5	10051.8	10780.5	11678.1	12156.1

Data displayed graphically, with 21= HR131; 45=HR154 and 51=HR160



Appendix to Chapter 4

PCR template layouts and photographs

Experiment 1

Date 11.3.99
Aim Preliminary white-rot fungus DNA extraction from soil
Treatment codes and description
100 – sterile soil
101 – sterile soil, wr45
102 – sterile soil, campy
103 – non sterile soil
104 – non sterile soil, wr45
105 – non sterile soil, campy
106 – sterile sand
107 - sterile sand, wr45
108 - sterile sand, campy
109 – non sterile sand
110 – non sterile sand, wr45
111 – non sterile sand, campy

soil = Tempelton silt loam (Simpsons Block), sterilised by autoclaving
sand = river sand, sterilised by autoclaving
campy = 100 µl of suspension from isolate 1958
fungus = wr45. 60 mg freeze dried weight

weigh air dry soil/sand (1 g) into sterile beat beating vial (with beads) and add fungus or campy or nothing for control and extract DNA in beat beater and purify DNA using biospin columns

PCR Template and Checklist

#	Code	DNA Source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buff (µl)
		#								
1	100	100	1	22.5	17	8	-	41	0.5	10
2	101	101	1	22.5	17	8	-	41	0.5	10
3	103	103	1	22.5	17	8	-	41	0.5	10
4	104	104	1	22.5	17	8	-	41	0.5	10
5	106	106	1	22.5	17	8	-	41	0.5	10
6	107	107	1	22.5	17	8	-	41	0.5	10
7	109	109	1	22.5	17	8	-	41	0.5	10
8	110	110	1	22.5	17	8	-	41	0.5	10
9	107-2	107	1	22.5	17	8	2	39	0.5	10
10	107-4	107	1	22.5	17	8	4	37	0.5	10
11	107-8	107	1	22.5	17	8	8	33	0.5	10
12	107-12	107	1	22.5	17	8	12	29	0.5	10
13	107-16	107	1	22.5	17	8	16	25	0.5	10
14	107-20	107	1	22.5	17	8	20	21	0.5	10
15	107-24	107	1	22.5	17	8	24	17	0.5	10
16	H_2O	H_2O	1	22.5	17	8	-	41	0.5	10
17	45	45	1	22.5	17	8	-	41	0.5	10
18										
19										
20										
		total		382.5	289	136			8.5	170

Final volume 100 µl
Added H_2O individually

MI: 382.5 + 289 + 136 = 807.5 ÷ 17 = 47.5 µl/vial
MII: 8.5 + 170 = 178.5 ÷17 = 10.5 µl/vial
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2 µl dye, 60 V, 1.5 h
Gel layout from left to right:
1 kb, 100, 101, 103, 104, 106, 107, 109, 110, 107-2,107-4, 107-8, 107-12, 107-16, 107-20, 107-24, H_2O, 45, 100 bp
no bands from PCR reaction displayed
(did not take photo)

run nested PCR
Nested PCR Template and Checklist

#	Code	DNA source PCR amplic	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buff (µl)
		#								
1	100	100	1	22.5	17	8	-	41	0.5	10
2	101	101	1	22.5	17	8	-	41	0.5	10
3	103	103	1	22.5	17	8	-	41	0.5	10
4	104	104	1	22.5	17	8	-	41	0.5	10
5	106	106	1	22.5	17	8	-	41	0.5	10
6	107	107	1	22.5	17	8	-	41	0.5	10
7	109	109	1	22.5	17	8	-	41	0.5	10
8	110	110	1	22.5	17	8	-	41	0.5	10
9	107-2	107-2	1	22.5	17	8	-	41	0.5	10
10	107-4	107-4	1	22.5	17	8	-	41	0.5	10
11	107-8	107-8	1	22.5	17	8	-	41	0.5	10
12	107-12	107-12	1	22.5	17	8	-	41	0.5	10
13	107-16	107-16	1	22.5	17	8	-	41	0.5	10
14	107-20	107-20	1	22.5	17	8	-	41	0.5	10
15	107-24	107-24	1	22.5	17	8	-	41	0.5	10
16	H_2O	H_2O	1	22.5	17	8	-	41	0.5	10
17	45	45	1	22.5	17	8	-	41	0.5	10
18	21	21	1	22.5	17	8	-	41	0.5	10
19	45	45	1	22.5	17	8	-	41	0.5	10
20	51	51	1	22.5	17	8	-	41	0.5	10
		total		450	340	160			10	200

Final volume 100 µl
Added H_2O individually
MI: 450 + 340 + 160 = 950 ÷ 20 = 47.5 µl/vial
MII: 10 + 200 = 210 ÷20 = 10.5 µl/vial
Row # 18, 19, 20 (21, 45, 51) amplicons were form PCR reaction on 27.1.99
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2 µl dye, 60 V, 1.5 h
Gel layout from left to right:
1 kb, 100, 101, 103, 104, 106, 107, 109, 110, 107-2,107-4, 107-8, 107-12, 107-16, 107-20, 107-24, H_2O, 45, 21, 45, 51, 21, 1 kb

c=H_2O

c=H_2O

Experiment 2

Date 8.7.99

Aim Fungal DNA extraction limit from soil (freeze dried mycelium)

Use freeze dried mycelium and 0.5 g non-sterile air dried soil (see experiment 3), for campy 0.125 ml of 8 x 10^7 cfu/ml was added to the soil (2 plates, scraped with 10 ml phosphate buffer)

Treatments

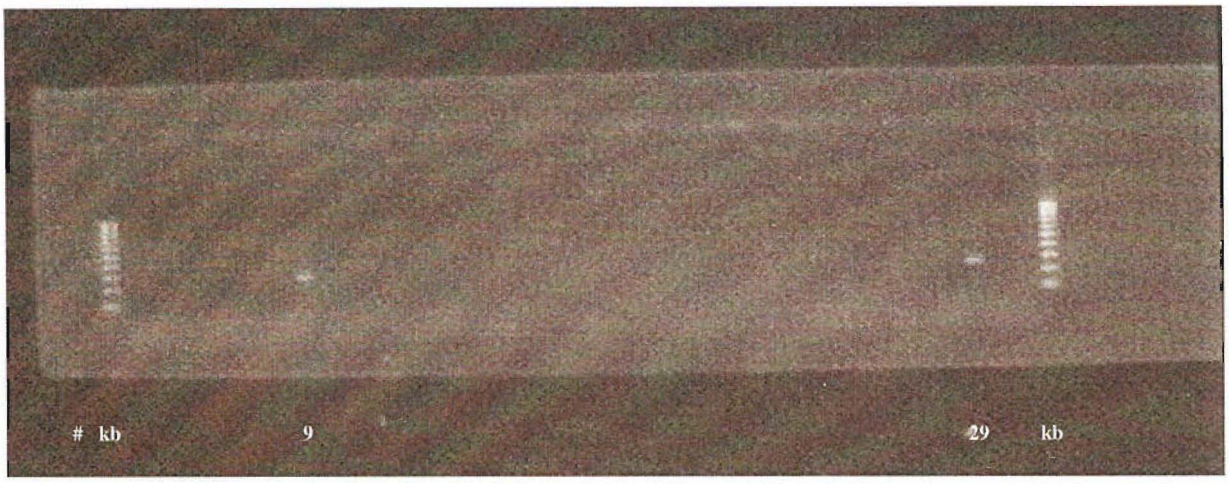
(Trt#- fungus,mycelium, soil)

120 – wr21, 30 mg, Tempelton
121 – wr21, 20 mg, Tempelton
122 – wr21, 10 mg, Tempelton
123 – wr21, 5 mg, Tempelton
124 – wr21, 2.5 mg, Tempelton
125 – control, 0 mg, Tempelton
126 – campy, 0.125 ml, Tempelton
127 – wr21, 20 mg, Sand
128 – wr21, 30 mg, Sand
129 – wr21, 10 mg, Sand
130 – wr21, 5 mg, Sand
131 – wr21, 2.5 mg, Sand
132 – control, 0 mg, Sand
133 – campy, 0.125 ml, Sand
134 – wr21, 30 mg, Broken River
135 – wr21, 20 mg, Broken River
136 – wr21, 10 mg, Broken River
137 – wr21, 5 mg, Broken River
138 – wr21, 2.5 mg, Broken River
139 – control, 0 mg, Broken River
140 – campy, 0.125 ml, Broken River
141 – wr45, 30 mg, Tempelton
142 – wr45, 30 mg, Sand
143 – wr45, 30 mg, Broken River
144 – wr51, 30 mg, Tempelton
145 – wr51, 30 mg, Sand
146 – wr51, 30 mg, Broken River

PCR Template and Checklist

#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buff (µl)
	#	#								
1	120	120	2	22.5	17	8	-	41	0.5	10
2	121	121	2	22.5	17	8	-	41	0.5	10
3	122	122	2	22.5	17	8	-	41	0.5	10
4	123	123	2	22.5	17	8	-	41	0.5	10
5	124	124	2	22.5	17	8	-	41	0.5	10
6	125	125	2	22.5	17	8	-	41	0.5	10
7	126	126	2	22.5	17	8	-	41	0.5	10
8	127	127	2	22.5	17	8	-	41	0.5	10
9	128	128	2	22.5	17	8	-	41	0.5	10
10	129	129	2	22.5	17	8	-	41	0.5	10
11	130	130	2	22.5	17	8	-	41	0.5	10
12	131	131	2	22.5	17	8	-	41	0.5	10
13	132	132	2	22.5	17	8	-	41	0.5	10
14	133	133	2	22.5	17	8	-	41	0.5	10
15	134	134	2	22.5	17	8	-	41	0.5	10
16	135	135	2	22.5	17	8	-	41	0.5	10
17	136	136	2	22.5	17	8	-	41	0.5	10
18	137	137	2	22.5	17	8	-	41	0.5	10
19	138	138	2	22.5	17	8	-	41	0.5	10
20	139	139	2	22.5	17	8	-	41	0.5	10
21	140	140	2	22.5	17	8	-	41	0.5	10
22	141	141	2	22.5	17	8	-	41	0.5	10
23	142	142	2	22.5	17	8	-	41	0.5	10
24	143	143	2	22.5	17	8	-	41	0.5	10
25	144	144	2	22.5	17	8	-	41	0.5	10
26	145	145	2	22.5	17	8	-	41	0.5	10
27	146	146	2	22.5	17	8	-	41	0.5	10
28	H2o	H2o	2	22.5	17	8	-	41	0.5	10
29	21	dna of gel	5	22.5	17	8	-	37	0.5	10
30	45	dna of gel	5	22.5	17	8	-	37	0.5	10
31	51	dna of gel	5	22.5	17	8	-	37	0.5	10
		total		697.5	527	248			15.5	310

Final volume 100 µl
Added H_2O individually
MI: 697.5 + 527 + 248 = 1472.5 ÷ 31 = 47.5 µl/vial
MII: 15.5 + 310 = 325.5 ÷ 31 = 10.5 µl/vial
Row # 29, 30, 31 was DNA from gel 9.7.99
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 12.5 µl PCR amplicon + 2 µl dye, 90 V, 1 h
Gel layout from left to right:
100 bp, 120, 121, 122, 123, 124, 125, 126, 127, 128, gap, 129, 130, 131, 132, 133, 134, gap, gap, 135, gap, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, gap, h2o, 21, 45, 51, 100 bp



Run nested PCR on PCR amplicon

Nested PCR Template and Checklist

#	Code	DNA source PCR amplic	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
	#	#								
1	O120	120	2.5	11.25	8.5	4	-	18.5	0.25	5
2	O121	121	2.5	11.25	8.5	4	-	18.5	0.25	5
3	O122	122	2.5	11.25	8.5	4	-	18.5	0.25	5
4	O123	123	2.5	11.25	8.5	4	-	18.5	0.25	5
5	o124	124	2.5	11.25	8.5	4	-	18.5	0.25	5
6	O125	125	2.5	11.25	8.5	4	-	18.5	0.25	5
7	O126	126	2.5	11.25	8.5	4	-	18.5	0.25	5
8	O127	127	2.5	11.25	8.5	4	-	18.5	0.25	5
9	O128	128	2.5	11.25	8.5	4	-	18.5	0.25	5
10	O129	129	2.5	11.25	8.5	4	-	18.5	0.25	5
11	O130	130	2.5	11.25	8.5	4	-	18.5	0.25	5
12	O131	131	2.5	11.25	8.5	4	-	18.5	0.25	5
13	O132	132	2.5	11.25	8.5	4	-	18.5	0.25	5
14	O133	133	2.5	11.25	8.5	4	-	18.5	0.25	5
15	O134	134	2.5	11.25	8.5	4	-	18.5	0.25	5
16	O135	135	2.5	11.25	8.5	4	-	18.5	0.25	5
17	O136	136	2.5	11.25	8.5	4	-	18.5	0.25	5
18	O137	137	2.5	11.25	8.5	4	-	18.5	0.25	5
19	O138	138	2.5	11.25	8.5	4	-	18.5	0.25	5
20	O139	139	2.5	11.25	8.5	4	-	18.5	0.25	5
21	O140	140	2.5	11.25	8.5	4	-	18.5	0.25	5
22	O141	141	2.5	11.25	8.5	4	-	18.5	0.25	5
23	O142	142	2.5	11.25	8.5	4	-	18.5	0.25	5
24	O143	143	2.5	11.25	8.5	4	-	18.5	0.25	5
25	O144	144	2.5	11.25	8.5	4	-	18.5	0.25	5
26	O145	145	2.5	11.25	8.5	4	-	18.5	0.25	5
27	O146	146	2.5	11.25	8.5	4	-	18.5	0.25	5
28	H2o	H2o	2.5	11.25	8.5	4	-	18.5	0.25	5
29	O21	21	2.5	11.25	8.5	4	-	18.5	0.25	5
30	O45	45	2.5	11.25	8.5	4	-	18.5	0.25	5
31	O51	51	2.5	11.25	8.5	4	-	18.5	0.25	5
32	21	dna of gel	2.5	11.25	8.5	4	-	18.5	0.25	5
		total		360	272	128		592	8	160

Final volume 50 µl

MI: 360+272+128+592 = 1352 ÷ 32 = 42.25 µl/vial

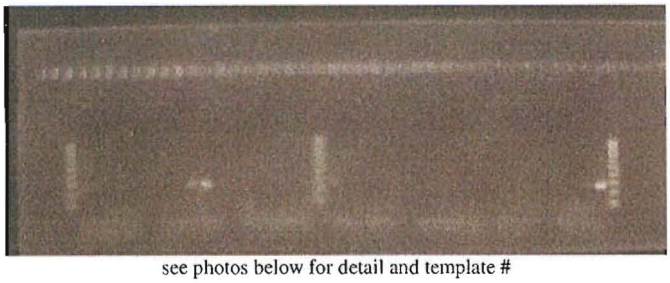
MII: 8+160 = 168 ÷ 32 = 5.25 µl/vial

Row # 32 was DNA from gel 9.7.99

Final primer concentration: 100 pmol

Prepare MI and MII, dispense MI and MII into PCR vials and finally add DNA template

Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2 µl dye, 90 V, 1 h
Gel layout from left to right:
100 bp, o120, o121, o122, o123, o124, gap, o125, o126, o127, o128, o129, gap, o130, o131, o132, o133, o134, 100 bp, o135, o136, o137, o138, o139, gap, o140, o141, o142, o143, o144, gap, 145, 146, h2o, gap, o21, o45, o51, 21, 100 bp



see photos below for detail and template #

Run PCR for campy
Protocol provided by Diana
Nested PCR Template and Checklist

#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) Pg50 5 pmol	Primer Reverse (µl) Pg3 5 pmol	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR fer (µl)
	#	#								
1	e125	125	2	0.5	0.5	4	3	34.5	0.5	5
2	e126	126	2	0.5	0.5	4	3	34.5	0.5	5
3	e132	132	2	0.5	0.5	4	3	34.5	0.5	5
4	e133	133	2	0.5	0.5	4	3	34.5	0.5	5
5	e139	139	2	0.5	0.5	4	3	34.5	0.5	5
6	e140	140	2	0.5	0.5	4	3	34.5	0.5	5
7	Control	Sel's DNA	2	0.5	0.5	4	3	34.5	0.5	5
8	H2o	H20	2	0.5	0.5	4	3	34.5	0.5	5
		total		4	4	32	24	276	4	40

Final volume 50 µl;
MI: 4+4+32+24+276 = 340 ÷ 8 = 42.25 µl/vial;
MII: 4+40 = 44 ÷ 8 = 5.5 µl/vial;
Final primer concentration: 0.01 pmol;
Prepare MI and MII, dispense MI and MII into PCR vials and finally add DNA template
Programme # 16
Gel electophoresis of PCR amplicon, using a 1-1.5% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h
Gel layout from left to right:
e125, e126, e132, e133, e139, e140, control, h2o, 100 bp

no photos taken

Experiment 3

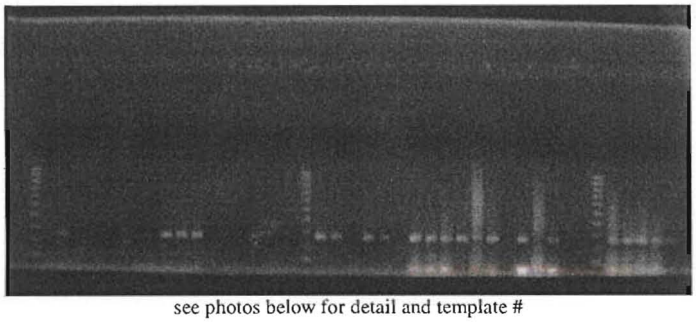
Date: 23.7.99
Aim: reproducibility of detection limits
Treatments:
150 – control, 0 mg, Tempelton
151 – wr21, 10 mg, Tempelton
152 – wr21, 20 mg, Tempelton
153 – wr21, 30 mg, Tempelton
154 – wr21, 60 mg, Tempelton
155 – wr21, 120 mg, Tempelton
156 – control, 0 mg, Sand
157 – wr21, 10 mg, Sand
158 – wr21, 20 mg, Sand
159 – wr21, 30 mg, Sand
160 – control, 0 mg, Broken River
161 – wr21, 30 mg, Broken River
162 – wr21, 60 mg, Broken River
163 – wr21, 120 mg, Broken River
164 – wr45, 60 mg, Tempelton
165 – wr45, 60 mg, Sand
166 – wr45, 60 mg, Broken River
167 – wr51, 60 mg, Tempelton
168 – wr51, 60 mg, Sand
169 – wr51, 60 mg, Broken River

PCR reaction
PCR Template and Checklist

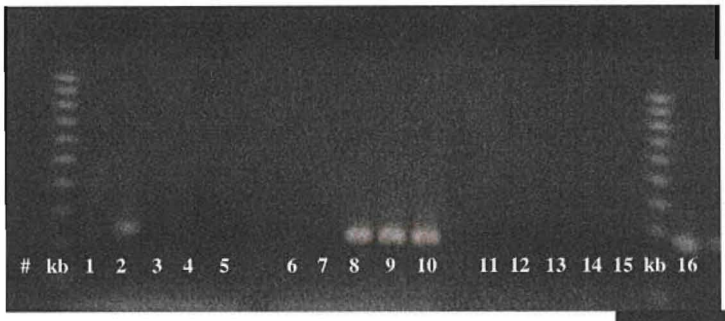
#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
	#	#								
1	x150	150	2	22.5	17	8	-	40	0.5	10
2	X151	151	2	22.5	17	8	-	40	0.5	10
3	X152	152	2	22.5	17	8	-	40	0.5	10
4	X153	153	2	22.5	17	8	-	40	0.5	10
5	X154	154	2	22.5	17	8	-	40	0.5	10
6	X155	155	2	22.5	17	8	-	40	0.5	10
7	X156	156	4	22.5	17	8	-	38	0.5	10
8	X157	157	2	22.5	17	8	-	40	0.5	10
9	X158	158	2	22.5	17	8	-	40	0.5	10
10	X159	159	2	22.5	17	8	-	40	0.5	10
11	X160	160	2	22.5	17	8	-	40	0.5	10
12	X161	161	2	22.5	17	8	-	40	0.5	10
13	X162	162	2	22.5	17	8	-	40	0.5	10
14	X163	163	2	22.5	17	8	-	40	0.5	10
15	X164	164	2	22.5	17	8	-	40	0.5	10
16	X165	165	2	22.5	17	8	-	40	0.5	10
17	X166	166	2	22.5	17	8	-	40	0.5	10
18	X167	167	2	22.5	17	8	-	40	0.5	10
19	X168	168	2	22.5	17	8	-	40	0.5	10
20	X169	169	2	22.5	17	8	-	40	0.5	10
21	A21	21	5	22.5	17	8	-	37	0.5	10
22	A45	45	5	22.5	17	8	-	37	0.5	10
23	A51	51	5	22.5	17	8	-	37	0.5	10
24	B21	21	5	22.5	17	8	-	37	0.5	10
25	B45	45	5	22.5	17	8	-	37	0.5	10
26	B51	51	5	22.5	17	8	-	37	0.5	10
27	C21	o21	5	22.5	17	8	-	37	0.5	10
28	C45	o45	5	22.5	17	8	-	37	0.5	10
29	C51	o51	5	22.5	17	8	-	37	0.5	10
30	D21	21	5	22.5	17	8	-	37	0.5	10
31	H2o	h2o	5	22.5	17	8	-	37	0.5	10
32	spare	-	-	22.5	17	8	-	-	0.5	10
		total		742.5	561	264			16	320

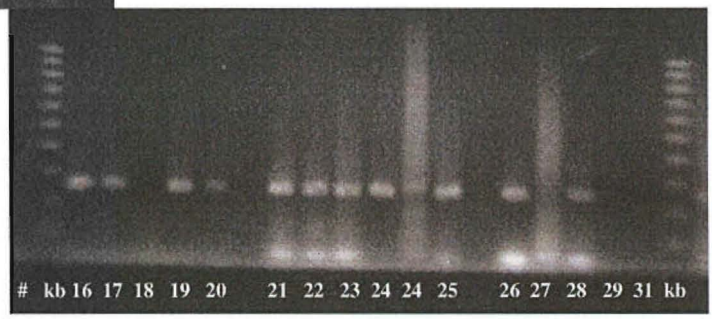
Final volume 100 µl

Added H_2O individually
MI: 742.5+561+264 = 1567.5 ÷ 32 = 47.5 µl/vial
MII: 16 + 320 = 336 ÷ 32 = 10.5 µl/vial
Row # 21, 22, 23 was purified DNA from 1.2.99, row # 24, 25, 26 was purified DNA from 9.7.99
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h
Gel layout from left to right:
100 bp, x150, x151, x152, x153, x154, gap, x155, x156, x157, x158, x159, gap, x160, x161, x162, x163, x164, 100 bp, x165, x166, x167, x168, x169, gap, a21, a45, a51, b21, b45, b51, gap, c21, c45, c51, d21, h2o, 100 bp, 17 (wr45), 18 (wr21), 19 (wr45), 20 (wr51), 8 (wr21)



see photos below for detail and template #



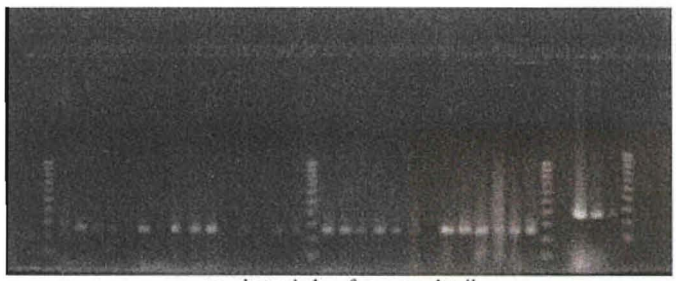


Run nested PCR on PCR amplicon
PCR Template and Checklist

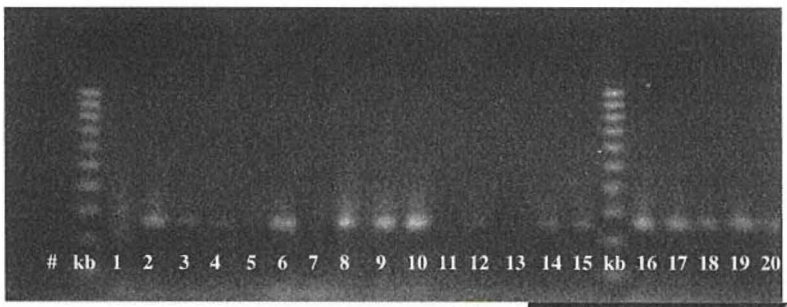
#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
	#	#								
1	xx150	x150	5	22.5	17	8	-	37	0.5	10
2	Xx151	x151	5	22.5	17	8	-	37	0.5	10
3	Xx152	X152	5	22.5	17	8	-	37	0.5	10
4	Xx153	X153	5	22.5	17	8	-	37	0.5	10
5	Xx154	X154	5	22.5	17	8	-	37	0.5	10
6	Xx155	X155	5	22.5	17	8	-	37	0.5	10
7	Xx156	X156	5	22.5	17	8	-	37	0.5	10
8	Xx157	X157	5	22.5	17	8	-	37	0.5	10
9	Xx158	X158	5	22.5	17	8	-	37	0.5	10
10	Xx159	X159	5	22.5	17	8	-	37	0.5	10

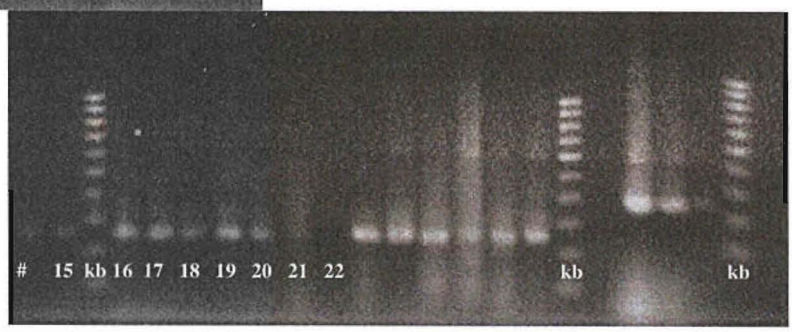
11	Xx160	X160	5	22.5	17	8	-	37	0.5	10
12	Xx161	X161	5	22.5	17	8	-	37	0.5	10
13	Xx162	X162	5	22.5	17	8	-	37	0.5	10
14	Xx163	X163	5	22.5	17	8	-	37	0.5	10
15	Xx164	X164	5	22.5	17	8	-	37	0.5	10
16	Xx165	X165	5	22.5	17	8	-	37	0.5	10
17	Xx166	X166	5	22.5	17	8	-	37	0.5	10
18	Xx167	X167	5	22.5	17	8	-	37	0.5	10
19	Xx168	X168	5	22.5	17	8	-	37	0.5	10
20	Xx169	X169	5	22.5	17	8	-	37	0.5	10
21	Aa21	A21	5	22.5	17	8	-	37	0.5	10
22	H2o	h2o	5	22.5	17	8	-	37	0.5	10
23	spare	-	-	22.5	17	8	-	-	0.5	10
		total		517.5	391	184			11.5	230

Final volume 100 µl
Added H_2O individually
MI: 517.5+391+184 = 1902.5 ÷ 23 = 47.5 µl/vial
MII: 11.5+230 = 241.5 ÷ 23 = 10.5 µl/vial
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon unless otherwise specified + 2.5 µl dye, 90 V, 1 h
Gel layout from left to right:
100 bp, xx150, xx151, xx152, xx153, xx154, xx155, xx156, xx157, xx158, xx159, xx160, xx161, xx162, xx163, xx164, 100 bp, xx165, xx166, xx167, xx168, xx169, aa21, h2o, (3 µl of the following) 21-1, 21-2, 45-1, 45-2, 51-1, 51-2, 100 bp, gap, (2 µl of the following) 21w2, 45w2, 51w2, 100 bp



see photos below for more detail





Summary
After 1st PCR only wr21, 30 mg in sand was detectable (120 series)
After nested PCR wr21, 30 and 20 mg in sand was detectable as well as 30, 20 and 5 mg in Tempelton soil (120 series)
Wr45 and wr53 were not detected at 30 mg (120 series) but were detected in the 150 series at 60 mg
Wr21 was detected in the 150 series using nested pcr at all concentrations and soils tested. Only in sand wr21 could be detected after 1st PCR.
This means there is an isolate effect on detection sensitivity

This also means there is a soil effect on detection.
Tempelton soil seems to have an inhibiting reaction on PCR. This is a common phenomenon and can be overcome by dilution.
Note that whilst soil and freeze dried mycelium volumes were halfed, final template was still recovered in 200 µl dd H_2O this explains the lack of detection of freeze dried mycelium at 30 mg using 0.5 g soil volumes

Experiment 4a

19.8.99
Aim: Extraction of DNA from fresh mycelium
Treatments
170 – wr21, 600 mg fresh mycelium, grown on PDA, 2 weeks at 30°C
171 – wr45, 600 mg fresh mycelium, grown on PDA, 2 weeks at 30°C
172 – wr51, 600 mg fresh mycelium, grown on PDA, 2 weeks at 30°C
173 – wr21 60 mg freeze dried mycelium
174 – wr45, 60 mg freeze dried mycelium
175 – wr51, 60 mg freeze dried mycelium

PCR Template and Checklist

#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
	#	#								
1	X170	170	1	22.5	17	8	-	41	0.5	10
2	X171	171	1	22.5	17	8	-	41	0.5	10
3	X172	172	1	22.5	17	8	-	41	0.5	10
4	X174	174	1	22.5	17	8	-	41	0.5	10
5	X175	175	1	22.5	17	8	-	41	0.5	10
6	X176	Xx158	1	22.5	17	8	-	41	0.5	10
7	Xh20	H20	1	22.5	17	8	-	41	0.5	10
8	X177	170	5	22.5	17	8	-	41	0.5	10
		total		180	136	64		328	4	80

Final volume 100 µl
MI: 180 + 136 + 64 + 328 = 708 ÷ 8 = 88.5 µl/vial
MII: 4 + 80 = 84 ÷ 8 = 10.5 µl/vial
Final primer concentration: 100 pmol
Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h
Gel layout from left to right:
100 bp, x170, x171, x173, x174, x175, x176, x177, x H_2O

Run nested PCR on PCR amplicon

PCR Template and Checklist

#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
	#	#								
1	Xx170	x170	5	22.5	17	8	-	37	0.5	10
2	Xx171	x171	5	22.5	17	8	-	37	0.5	10
3	Xx172	x172	5	22.5	17	8	-	37	0.5	10
4	Xx174	x174	5	22.5	17	8	-	37	0.5	10
5	Xx175	x175	5	22.5	17	8	-	37	0.5	10
6	Xx176	X176	5	22.5	17	8	-	37	0.5	10
7	H_2O	H_2O	5	22.5	17	8	-	37	0.5	10
8	Xx177	x177	5	22.5	17	8	-	37	0.5	10
		total		180	136	64		296	4	80

Final volume 100 µl

MI: 180 + 136 + 64 + 296 = 708 ÷ 8 = 84.5 µl/vial

MII: 4 + 80 = 84 ÷ 8 = 10.5 µl/vial

Final primer concentration: 100 pmol

Prepare MI and MII, dispense H_2O and MI and MII into PCR vials and finally add DNA template

Programme # 10

Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h

Gel lay out from left to right:

100 bp, xx170, xx171, xx173, xx174, xx175, x1x76, xx177, H_2O

Note: Because photographing unit was changed no photos could be taken on the day, but gel was left in fridge for one week prior to photography, hence the bands diffused! Therefore gel was run again in combination with the 190 series (experiment 8)

Experiment 4b

30.8.99

Aim: Detection limit of fresh mycelium form soil

Treatments

190 – wr21, 0.6 g fresh mycelium, Tempelton soil (0.5 g, air dry)
191 – wr21, 0.3 g fresh mycelium, Tempelton soil (0.5 g, air dry)
192 – wr21, 0.06 g fresh mycelium, Tempelton soil (0.5 g, air dry)
193 – wr21, 0.6 g fresh mycelium, Sand (0.5 g, air dry)
194 – wr21, 0.3 g fresh mycelium, Sand (0.5 g, air dry)
195 – wr21, 0.06 g fresh mycelium, Sand (0.5 g, air dry)
196 – wr21, 0.6 g fresh mycelium, Broken River (0.5 g, air dry)
197 – wr21, 0.3 g fresh mycelium, Broken River (0.5 g, air dry)
198 – wr21, 0.06 g fresh mycelium, Broken River (0.5 g, air dry)
199 – wr45, 0.6 g fresh mycelium, Tempelton soil (0.5 g, air dry)
200 – wr45, 0.6 g fresh mycelium, Sand (0.5 g, air dry)
201 – wr45, 0.6 g fresh mycelium, Broken River (0.5 g, air dry)
202 – wr51, 0.6 g fresh mycelium, Tempelton soil (0.5 g, air dry)
203 – wr51, 0.6 g fresh mycelium, Sand (0.5 g, air dry)
204 – wr51, 0.6 g fresh mycelium, Broken River (0.5 g, air dry)

PCR reaction

PCR Template and Checklist

#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
1	x190	190	1	22.5	17	8	-	41	0.5	10
2	X191	191	1	22.5	17	8	-	41	0.5	10
3	X192	192	1	22.5	17	8	-	41	0.5	10
4	X193	193	1	22.5	17	8	-	41	0.5	10
5	X194	194	1	22.5	17	8	-	41	0.5	10
6	X195	195	1	22.5	17	8	-	41	0.5	10
7	X196	196	1	22.5	17	8	-	41	0.5	10
8	X197	197	1	22.5	17	8	-	41	0.5	10
9	X198	198	1	22.5	17	8	-	41	0.5	10

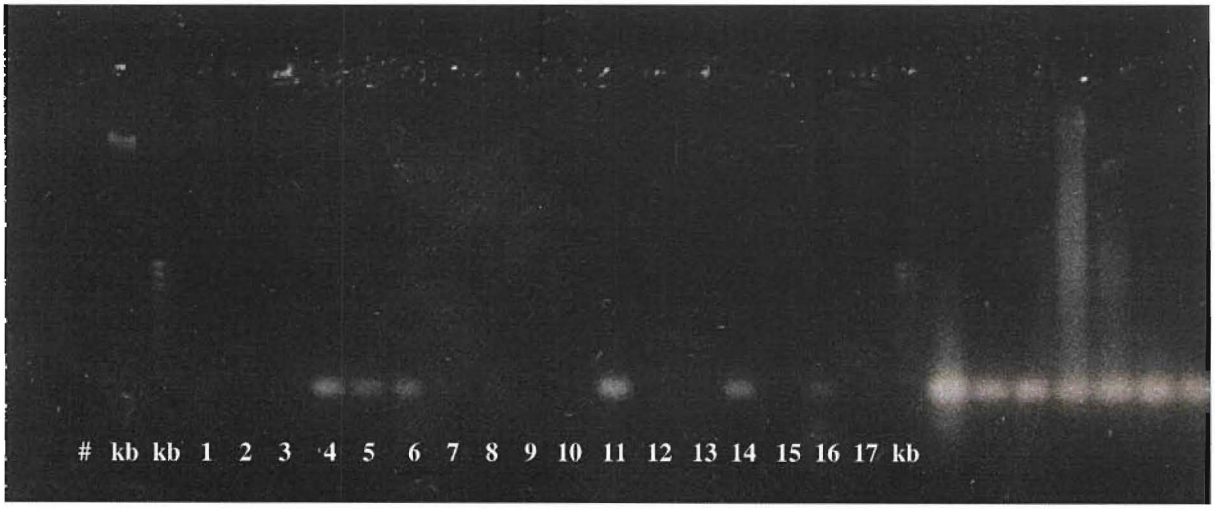
10	X199	199	1	22.5	17	8	-	41	0.5	10
11	X200	200	1	22.5	17	8	-	41	0.5	10
12	X201	201	1	22.5	17	8	-	41	0.5	10
13	X202	202	1	22.5	17	8	-	41	0.5	10
14	X203	203	1	22.5	17	8	-	41	0.5	10
15	X204	204	1	22.5	17	8	-	41	0.5	10
16	21con	Xx158	1	22.5	17	8	-	41	0.5	10
17	H_2O	H_2O	1	22.5	17	8	-	41	0.5	10
18	spare	-	-	22.5	17	8	-	41	0.5	10
		total		405	306	144		738	9	180

MI: 405 + 306 +144 + 738 = 1593 ÷ 18 = 88.5 µl/vial
MII: 9 + 180 = 189 ÷ 18 = 10.5 µl/vial
Final volume 100 µl; Final primer concentration: 100 pmol, Prepare MI and MII, dispense MI and MII into PCR vials and finally add DNA template; Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h in combination with nested PCR product

Run nested PCR on PCR amplicon
PCR Template and Checklist

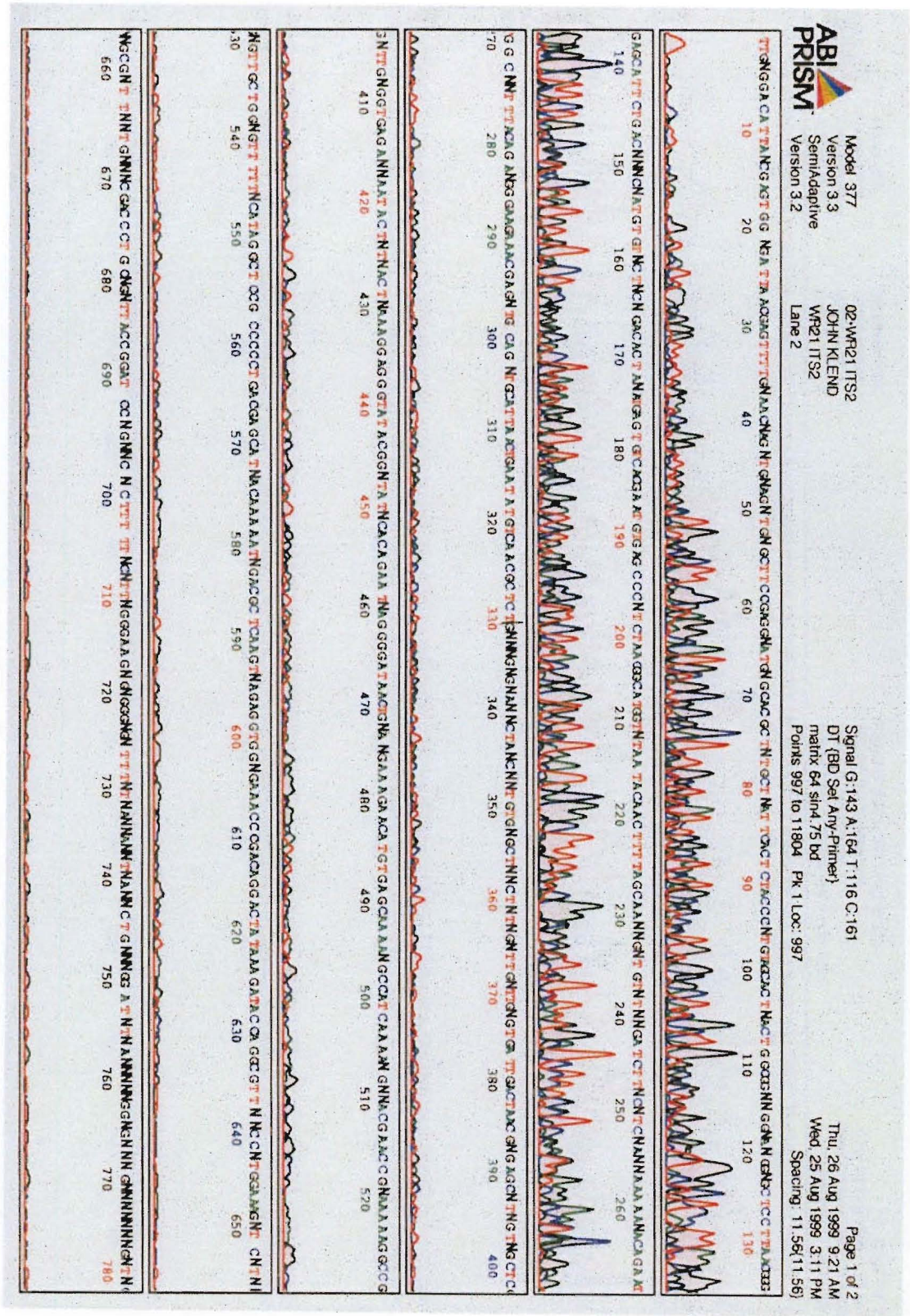
#	Code	DNA source	PCR/DNA Template (µl)	Primer Forward (µl) ITS-1	Primer Reverse (µl) ITS-2	dNTP 0.2 mM (µl)	MgCl 1.5 mM (µl)	dd H_2O (µl)	Taq (µl)	10x PCR buffer (µl)
1	xx190	X190	2.5	11.25	8.5	4	-	18.5	0.25	5
2	Xx191	X191	2.5	11.25	8.5	4	-	18.5	0.25	5
3	Xx192	X192	2.5	11.25	8.5	4	-	18.5	0.25	5
4	Xx193	X193	2.5	11.25	8.5	4	-	18.5	0.25	5
5	Xx194	X194	2.5	11.25	8.5	4	-	18.5	0.25	5
6	Xx195	X195	2.5	11.25	8.5	4	-	18.5	0.25	5
7	Xx196	X196	2.5	11.25	8.5	4	-	18.5	0.25	5
8	Xx197	X197	2.5	11.25	8.5	4	-	18.5	0.25	5
9	Xx198	X198	2.5	11.25	8.5	4	-	18.5	0.25	5
10	Xx199	X199	2.5	11.25	8.5	4	-	18.5	0.25	5
11	Xx200	X200	2.5	11.25	8.5	4	-	18.5	0.25	5
12	Xx201	X201	2.5	11.25	8.5	4	-	18.5	0.25	5
13	Xx202	X202	2.5	11.25	8.5	4	-	18.5	0.25	5
14	Xx203	X203	2.5	11.25	8.5	4	-	18.5	0.25	5
15	Xx204	X204	2.5	11.25	8.5	4	-	18.5	0.25	5
16	21con	Xx158	2.5	11.25	8.5	4	-	18.5	0.25	5
17	H_2O	H_2O	2.5	11.25	8.5	4	-	18.5	0.25	5
18	Spare	-	-	11.25	8.5	4	-	18.5	0.25	5
		total		202.5	153	72		333	4.5	90

Final volume 50 µl
MI: 202.5 + 153 + 72 + 333 = 760.5 ÷ 18 = 42.25 µl/vial
MII: 4.5 + 90 = 94.5 ÷ 18 = 5.25 µl/vial
Final primer concentration: 100 pmol
Prepare MI and MII, dispense MI and MII into PCR vials and finally add DNA template
Programme # 10
Gel electophoresis of PCR amplicon, using a 2% agarose gel, 10 µl PCR amplicon + 2.5 µl dye, 100 V, 1 h in combination with PCR product
Gel layout from left to right:
1 kb+, 100 bp, x190, x191, x192, x193, x194, x195, x196, x197, x198, x199, x200, x201, x202, x203, x204, 21con, H_2O, 100 bp, xx190, xx191, xx192, xx193, xx194, xx195, xx196, xx197, xx198, xx199, xx200, xx201, xx202, xx203, xx204, 21con, H_2O, 100 bp, x170, x171, x172, x174, x175, x176, x177, H_2O, 100 pb, xx170, xx171, xx172, xx174, xx175, xx176, xx177, H_2O, 100 bp


kb kb 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 kb

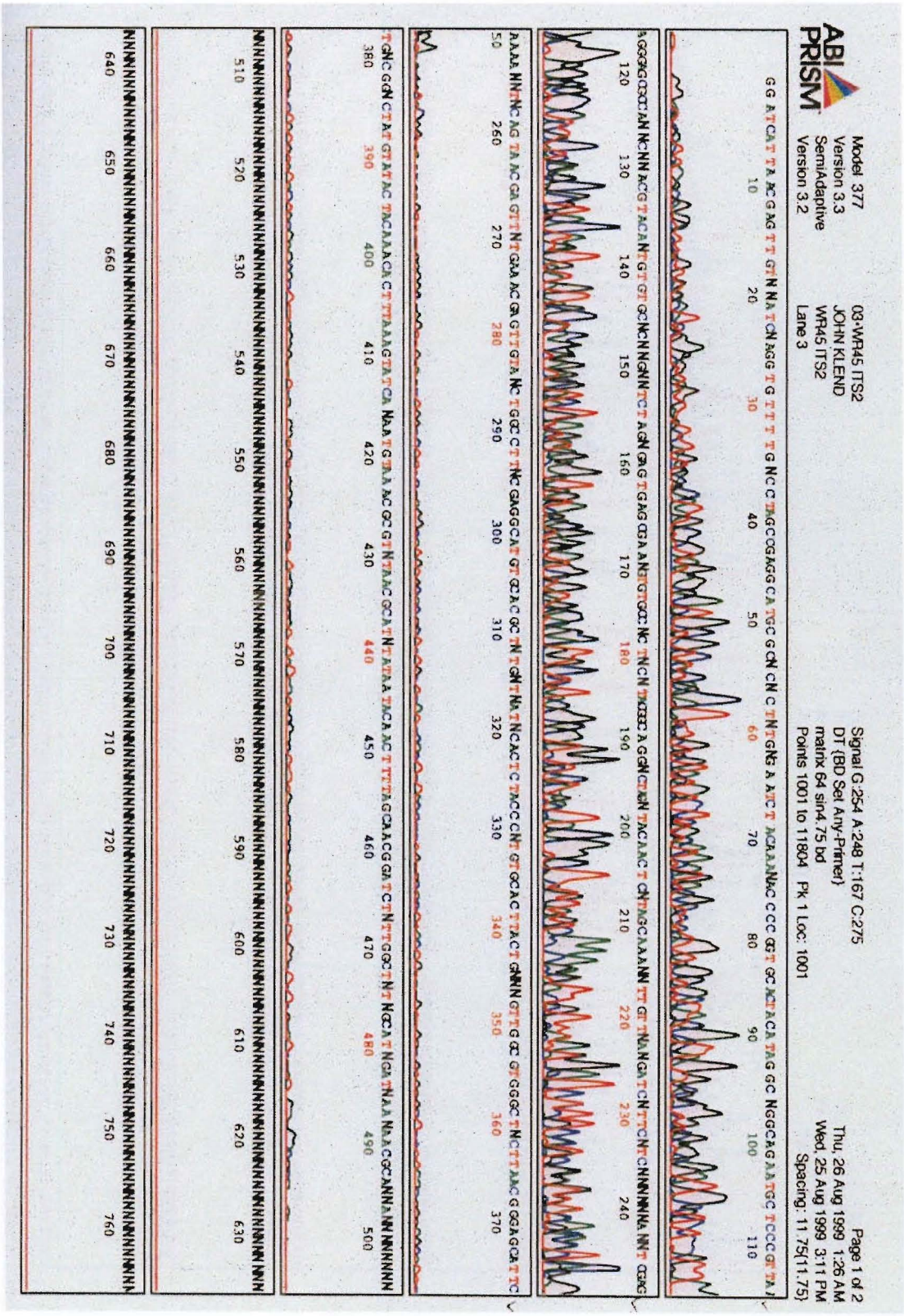
Automated sequencing results

For *Trametes versicolor* isolate WR21=HR131



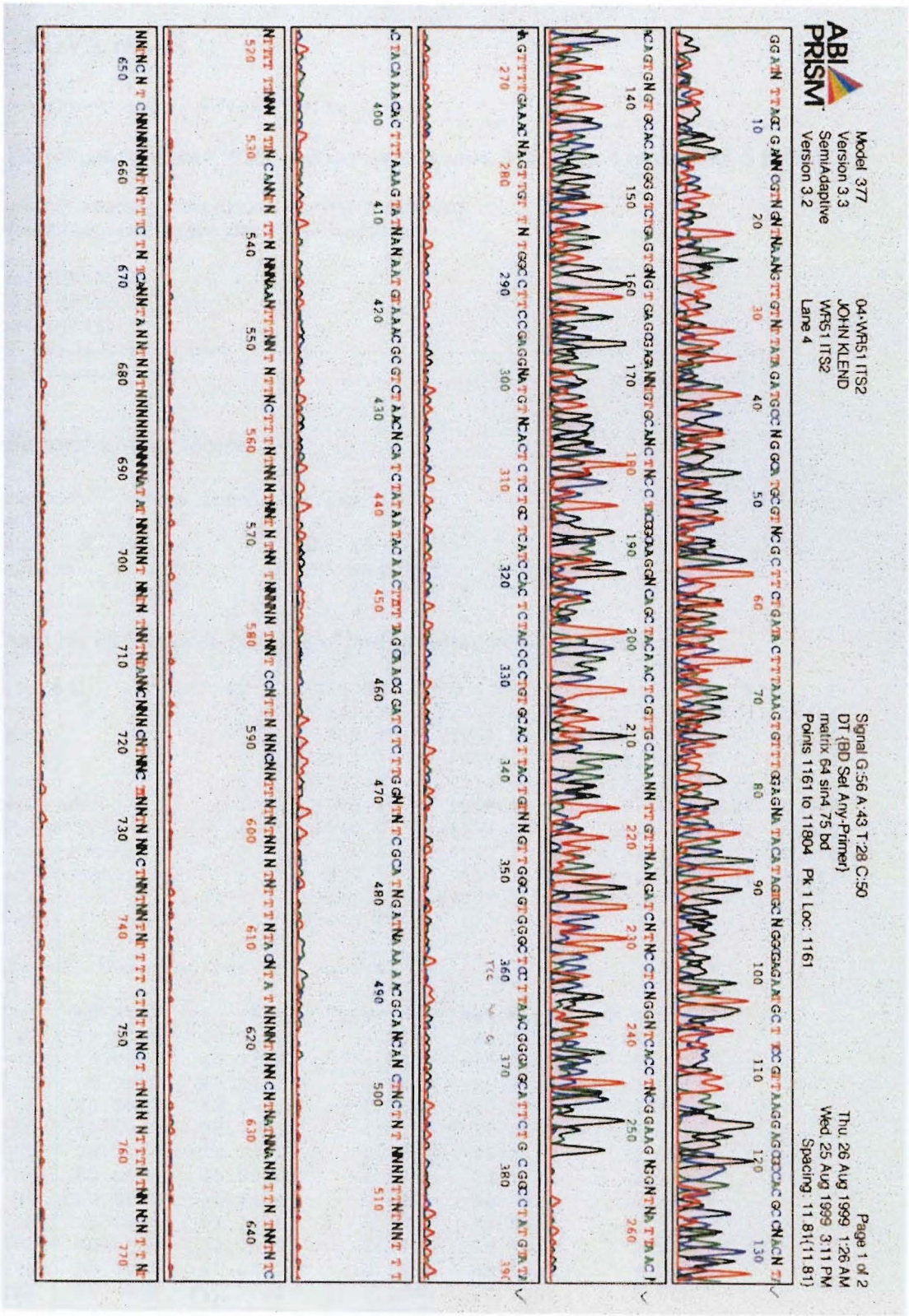
Automated sequencing results

For *Trametes versicolor* isolate WR45=HR134



Automated sequencing results

For *Trametes versicolor* isolate WR51=HR160



Apendix to Chapter 5

ANOVA tables

```
Worksheet size: 100000 cells
```

Experimental data - WRF soil survival: 2 soils, 3 isolates, 4 moistures, 5 SCS

pplate7= percentage area covered after 7 days incubated
density7=mycelial density after 7 days incubation

wr21=HR131
wr51=HR160
wr45=HR154
d= Temuka deep clay loam
s= Wakanui silt loam

General Linear Model

```
Factor      Type Levels Values
soil       fixed      2 d s
whc        fixed      4  40  60  80 100
strain     fixed      3 wr21 wr45 wr51
scs        fixed      5   0  25  50  75 100
```

Analysis of Variance for pplate7, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
soil	1	35581.3	33703.0	33703.0	136.03	0.000
whc	3	10638.3	10463.9	3488.0	14.08	0.000
strain	2	2841.0	3556.1	1778.1	7.18	0.001
scs	4	24991.6	24974.0	6243.5	25.20	0.000
soil*whc	3	4778.9	3866.4	1288.8	5.20	0.002
whc*strain	6	1204.0	1197.0	199.5	0.81	0.567
soil*strain	2	3971.8	4147.6	2073.8	8.37	0.000
soil*whc*strain	6	539.3	539.3	89.9	0.36	0.902
Error	274	67887.3	67887.3	247.8		
Total	301	152433.7				

Unusual Observations for pplate7

Obs	pplate7	Fit	StDev Fit	Residual	St Resid
7	5.0000	40.7112	4.8372	-35.7112	-2.38R
8	5.0000	40.7112	4.8372	-35.7112	-2.38R
9	5.0000	40.7112	4.8372	-35.7112	-2.38R
12	80.0000	39.1809	5.8090	40.8191	2.79R
50	60.0000	28.7642	4.8395	31.2358	2.09R
51	70.0000	28.7642	4.8395	41.2358	2.75R
89	80.0000	45.8476	4.8395	34.1524	2.28R
90	80.0000	45.8476	4.8395	34.1524	2.28R
98	5.0000	38.2455	4.8372	-33.2455	-2.22R
105	70.0000	31.1957	4.8475	38.8043	2.59R
108	90.0000	41.4457	4.8475	48.5543	3.24R
123	80.0000	41.2642	4.8395	38.7358	2.59R
124	80.0000	47.9309	4.8395	32.0691	2.14R

135	10.0000	40.3289	4.8372	-30.3289	-2.02R
139	70.0000	36.8623	4.8475	33.1377	2.21R
143	80.0000	43.5290	4.8475	36.4710	2.44R
144	80.0000	43.5290	4.8475	36.4710	2.44R
165	50.0000	14.0327	5.2803	35.9673	2.43R
188	60.0000	18.1847	4.7348	41.8153	2.79R
305	50.0000	14.0994	4.4298	35.9006	2.38R
317	50.0000	16.7133	5.2346	33.2867	2.24R

R denotes an observation with a large standardized residual.

Analysis of Variance for density7, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
soil	1	3.8269	5.2568	5.2568	5.83	0.016
whc	3	43.7790	38.4107	12.8036	14.19	0.000
strain	2	30.7202	27.7164	13.8582	15.36	0.000
scs	4	83.9584	83.5058	20.8764	23.14	0.000
soil*whc	3	4.8576	5.1722	1.7241	1.91	0.128
whc*strain	6	10.5523	10.0369	1.6728	1.85	0.089
soil*strain	2	5.6497	5.6793	2.8397	3.15	0.045
soil*whc*strain	6	3.5994	3.5994	0.5999	0.66	0.678
Error	274	247.2154	247.2154	0.9022		
Total	301	434.1589				

Unusual Observations for density7

Obs	density7	Fit	StDev Fit	Residual	St Resid
35	5.00000	2.96881	0.29253	2.03119	2.25R
47	1.00000	2.99353	0.29204	-1.99353	-2.21R
49	1.00000	2.99353	0.29204	-1.99353	-2.21R
66	5.00000	3.13548	0.29253	1.86452	2.06R
187	5.00000	3.02232	0.28573	1.97768	2.18R
188	5.00000	3.02232	0.28573	1.97768	2.18R
189	5.00000	3.02232	0.28573	1.97768	2.18R
197	5.00000	1.87207	0.26732	3.12793	3.43R
224	1.00000	3.09656	0.26831	-2.09656	-2.30R
238	3.00000	0.93874	0.26732	2.06126	2.26R
241	5.00000	2.67207	0.26732	2.32793	2.55R
242	5.00000	2.67207	0.26732	2.32793	2.55R
243	5.00000	2.67207	0.26732	2.32793	2.55R
254	5.00000	2.91300	0.30447	2.08700	2.32R
273	1.00000	2.81422	0.31679	-1.81422	-2.03R
281	0.00000	2.52459	0.30735	-2.52459	-2.81R
282	0.00000	2.52459	0.30735	-2.52459	-2.81R
287	5.00000	3.07207	0.26732	1.92793	2.12R

R denotes an observation with a large standardized residual.

Worksheet size: 100000 cells
Retrieving project from file:
C:\AMONIKA\PROJECTS\BIOREMEDIATION\MONIKA\LINCOLN SOIL SURVIVAL EXPERIMENTS\BIOMED- WRF SOIL SURVIVAL\EXP1.MPJ

Experimental data - WRF soil survival: PCP effect

Analysis of Variance (Balanced Designs)

Factor	Type	Levels	Values				
pcp ppm	fixed	5	0	20	50	100	200
fungi	fixed	3	21	45	51		

Analysis of Variance for 7area

Source	DF	SS	MS	F	P
pcp ppm	4	37938.3	9484.6	189.69	0.000
fungi	2	411.7	205.8	4.12	0.038
pcp ppm*fungi	8	996.7	124.6	2.49	0.061
Error	15	750.0	50.0		
Total	29	40096.7			

Analysis of Variance for 7dens

Source	DF	SS	MS	F	P
pcp ppm	4	6.133	1.533	0.72	0.592
fungi	2	26.667	13.333	6.25	0.011
pcp ppm*fungi	8	26.667	3.333	1.56	0.217
Error	15	32.000	2.133		
Total	29	91.467			

Analysis of Variance for 14area

Source	DF	SS	MS	F	P
pcp ppm	4	34478.3	8619.6	19.23	0.000
fungi	2	3695.0	1847.5	4.12	0.037
pcp ppm*fungi	8	8471.7	1059.0	2.36	0.072
Error	15	6725.0	448.3		
Total	29	53370.0			

Analysis of Variance for 14dens

Source	DF	SS	MS	F	P
pcp ppm	4	10.467	2.617	1.19	0.355
fungi	2	18.867	9.433	4.29	0.034
pcp ppm*fungi	8	15.133	1.892	0.86	0.569
Error	15	33.000	2.200		
Total	29	77.467			

Analysis of Variance for 21area

Source	DF	SS	MS	F	P
pcp ppm	4	32150.0	8037.5	13.16	0.000
fungi	2	2795.0	1397.5	2.29	0.136
pcp ppm*fungi	8	13330.0	1666.2	2.73	0.045
Error	15	9162.5	610.8		
Total	29	57437.5			

Analysis of Variance for 21dens

Source	DF	SS	MS	F	P
pcp ppm	4	4.533	1.133	0.64	0.641
fungi	2	9.800	4.900	2.77	0.094
pcp ppm*fungi	8	11.867	1.483	0.84	0.583
Error	15	26.500	1.767		
Total	29	52.700			

Analysis of Variance for 28area

Source	DF	SS	MS	F	P
pcp ppm	4	36533.3	9133.3	13.22	0.000
fungi	2	5611.7	2805.8	4.06	0.039
pcp ppm*fungi	8	10996.7	1374.6	1.99	0.119
Error	15	10362.5	690.8		
Total	29	63504.2			

Analysis of Variance for 28dens

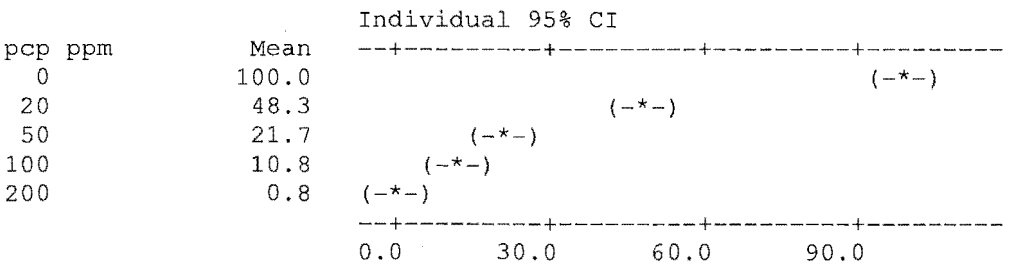
Source	DF	SS	MS	F	P
pcp ppm	4	36.867	9.217	2.66	0.074
fungi	2	2.600	1.300	0.38	0.694
pcp ppm*fungi	8	53.733	6.717	1.94	0.128
Error	15	52.000	3.467		
Total	29	145.200			

```
Saving file as: C:\aMonika\projects\Bioremediation\monika\lincoln soil
survival experiments\BIOMED- WRF soil survival\PCP colonisation.MPJ
* NOTE  * Existing file replaced.
```

Two-way Analysis of Variance

Analysis of Variance for 7area

Source	DF	SS	MS	F	P
pcp ppm	4	37938.3	9484.6	189.69	0.000
fungi	2	411.7	205.8	4.12	0.038
Interaction	8	996.7	124.6	2.49	0.061
Error	15	750.0	50.0		
Total	29	40096.7			



```
                        Individual 95% CI
fungi           Mean    -------+---------+---------+---------+----
21              40.5                      (---------*---------)
45              31.5    (---------*---------)
51              37.0              (---------*---------)
                        -------+---------+---------+---------+----
                            30.0      35.0      40.0      45.0
```

Analysis of Variance (Balanced Designs)

```
Factor     Type Levels Values
pcpmg/kg   fixed      5      0    20    50   100   200
isolate    fixed      3     21    45    51
day        fixed      4      7    14    21    28
```

```
Analysis of Variance for area

Source       DF         SS         MS        F      P
pcpmg/kg      4     136772      34193    55.79  0.000
isolate       2      10222       5111     8.34  0.000
day           3       4891       1630     2.66  0.052
Error       110      67415        613
Total       119     219299
```

```
Analysis of Variance for density

Source       DF         SS         MS        F      P
pcpmg/kg      4     18.950      4.738     1.65  0.168
isolate       2     31.400     15.700     5.46  0.006
day           3     42.492     14.164     4.92  0.003
Error       110    316.483      2.877
Total       119    409.325
```

Two-way Analysis of Variance

```
Analysis of Variance for area
Source         DF         SS         MS         F         P
pcpmg/kg        4     136772      34193     72.60     0.000
isolate         2      10222       5111     10.85     0.000
Interaction     8      22855       2857      6.07     0.000
Error         105      49450        471
Total         119     219299
```

```
                        Individual 95% CI
pcpmg/kg         Mean   ---+---------+---------+---------+--------
  0             100.0                                    (--*--)
 20              65.2                          (--*--)
 50              35.8                 (--*--)
100              26.5              (--*--)
200               2.1     (--*--)
                        ---+---------+---------+---------+--------
                         0.0       30.0      60.0      90.0
```

```
                        Individual 95% CI
isolate         Mean    ----+---------+---------+---------+-------
21              50.8                        (------*------)
45              33.0    (------*------)
51              54.0                              (------*------)
                        ----+---------+---------+---------+-------
                         30.0       40.0       50.0       60.0
```

Analysis of Variance (Balanced Designs)

```
Factor      Type Levels Values
pcpmg/kg   fixed      5      0     20     50    100    200
isolate    fixed      3     21     45     51
day        fixed      4      7     14     21     28
```

Analysis of Variance for area

Source	DF	SS	MS	F	P
pcpmg/kg	4	136772.1	34193.0	75.70	0.000
isolate	2	10221.7	5110.8	11.32	0.000
day	3	4890.8	1630.3	3.61	0.017
pcpmg/kg*isolate	8	22855.4	2856.9	6.33	0.000
pcpmg/kg*day	12	4327.9	360.7	0.80	0.651
isolate*day	6	2291.7	381.9	0.85	0.538
Error	84	37939.6	451.7		
Total	119	219299.2			

Analysis of Variance for density

Source	DF	SS	MS	F	P
pcpmg/kg	4	18.950	4.738	1.96	0.108
isolate	2	31.400	15.700	6.49	0.002
day	3	42.492	14.164	5.86	0.001
pcpmg/kg*isolate	8	47.850	5.981	2.47	0.018
pcpmg/kg*day	12	39.050	3.254	1.35	0.209
isolate*day	6	26.533	4.422	1.83	0.103
Error	84	203.050	2.417		
Total	119	409.325			

Analysis of Variance (Balanced Designs)

```
Factor      Type Levels Values
pcpmg/kg   fixed      5      0     20     50    100    200
isolate    fixed      3     21     45     51
day        fixed      4      7     14     21     28
```

Analysis of Variance for area

Source	DF	SS	MS	F	P
pcpmg/kg	4	136772.1	34193.0	75.98	0.000
isolate	2	10221.7	5110.8	11.36	0.000
day	3	4890.8	1630.3	3.62	0.018

pcpmg/kg*isolate	8	22855.4	2856.9	6.35	0.000
pcpmg/kg*day	12	4327.9	360.7	0.80	0.647
isolate*day	6	2291.7	381.9	0.85	0.538
pcpmg/kg*isolate*day	24	10939.6	455.8	1.01	0.466
Error	60	27000.0	450.0		
Total	119	219299.2			

Analysis of Variance for density

Source	DF	SS	MS	F	P
pcpmg/kg	4	18.950	4.738	1.98	0.109
isolate	2	31.400	15.700	6.56	0.003
day	3	42.492	14.164	5.92	0.001
pcpmg/kg*isolate	8	47.850	5.981	2.50	0.021
pcpmg/kg*day	12	39.050	3.254	1.36	0.210

Apendix to Chapter 6

Soil colonisation data

With 45 = HR154, 21 = HR131, 51 = HR160 and –1= missing data and/or not included in this sub-experiment

Expt no	Trt no	wr	soil	whc	scs	rep	7days (%)	7 days dens	14days (%)	14 days dens	21days (%)	21 days dens
1	1		45pumice	100	25	1		40m		70h		100h
1	1		45pumice	100	25	2		10l		40l		100m
1	2		45pumice	80	25	1		70m		100h		100h
1	2		45pumice	80	25	2		70m		100h		100h
1	3		45pumice	60	25	1		80l		60m		90m
1	3		45pumice	60	25	2		60l		80m		90m
1	4		45pumice	40	25	1		50l		50m		70m
1	4		45pumice	40	25	2		50l		60m		70m
1	5		51pumice	100	25	1		90m		100h		100h
1	5		51pumice	100	25	2		90m		100h		100h
1	6		51pumice	80	25	1		80m		100h		100h
1	6		51pumice	80	25	2		100m		100h		100h
1	7		51pumice	60	25	1		100l		100m		100h
1	7		51pumice	60	25	2		80l		80m		90m
1	8		51pumice	40	25	1		30l		30l		40m
1	8		51pumice	40	25	2		40l		30l		50m
1	9		21pumice	100	25	1		60m		100m		100h
1	9		21pumice	100	25	2		30m		60m		100h
1	10		21pumice	80	25	1		90m		100m		100h
1	10		21pumice	80	25	2		90m		100m		100h
1	11		21pumice	60	25	1		50m		40m		60m
1	11		21pumice	60	25	2		90m		90m		90m
1	12		21pumice	40	25	1		60l		60m		50m
1	12		21pumice	40	25	2		70l		70m		60m
1	13		45ultic	100	25	1		60l		100m		100h
1	13		45ultic	100	25	2		30l		30m		100m
1	14		45ultic	80	25	1		100l		40m		100m
1	14		45ultic	80	25	2		100l		50m		100m
1	15		45ultic	60	25	1		50l		50l		40l
1	15		45ultic	60	25	2		50l		50l		50l
1	16		45ultic	40	25	1		5l		5l		10l
1	16		45ultic	40	25	2		5l		5l		20l
1	17		51ultic	100	25	1		70m		100m		100h
1	17		51ultic	100	25	2		70m		70m		100h
1	18		51ultic	80	25	1		90m		80m		90m
1	18		51ultic	80	25	2		80m		80m		80m
1	19		51ultic	60	25	1		60l		60l		60l
1	19		51ultic	60	25	2		20l		20l		20l
1	20		51ultic	40	25	1		5l		5l		10l
1	20		51ultic	40	25	2		10l		10l		10l
1	21		21ultic	100	25	1		80l		90h		100h
1	21		21ultic	100	25	2		80l		90h		100h

1	22	21ultic	80	25	1	70l	80l	100l
1	22	21ultic	80	25	2	60m	70m	100m
1	23	21ultic	60	25	1	30l	20l	40m
1	23	21ultic	60	25	2	30l	20l	30l
1	24	21ultic	40	25	1	5l	5l	5l
1	24	21ultic	40	25	2	5l	5l	5l
1	25	45granular	100	25	1	40l	80m	100m
1	25	45granular	100	25	2	20l	70m	100m
1	26	45granular	80	25	1	80l	60l	100m
1	26	45granular	80	25	2	90l	90l	100m
1	27	45granular	60	25	1	50l	50l	70m
1	27	45granular	60	25	2	90l	80l	80m
1	28	45granular	40	25	1	10l	10l	20m
1	28	45granular	40	25	2	10l	10l	20l
1	29	51granular	100	25	1	20m	50m	100h
1	29	51granular	100	25	2	40m	60m	100h
1	30	51granular	80	25	1	40l	60m	100m
1	30	51granular	80	25	2	40l	40m	100m
1	31	51granular	60	25	1	60l	50l	70m
1	31	51granular	60	25	2	50l	50l	50m
1	32	51granular	40	25	1	10l	20l	30m
1	32	51granular	40	25	2	10l	10l	20m
1	33	21granular	100	25	1	10l	20l	100h
1	33	21granular	100	25	2	10l	20l	100h
1	34	21granular	80	25	1	10l	10l	90l
1	34	21granular	80	25	2	10l	10l	100l
1	35	21granular	60	25	1	10l	20l	40l
1	35	21granular	60	25	2	10l	30l	30l
1	36	21granular	40	25	1	10l	10l	30l
1	36	21granular	40	25	2	20l	10l	30l
1	37	45allophanic	100	25	1	20m	50m	90m
1	37	45allophanic	100	25	2	20m	50m	100m
1	38	45allophanic	80	25	1	60m	80h	100h
1	38	45allophanic	80	25	2	50m	60h	100h
1	39	45allophanic	60	25	1	50m	50h	90h
1	39	45allophanic	60	25	2	50m	70h	90h
1	40	45allophanic	40	25	1	20l	20l	40m
1	40	45allophanic	40	25	2	30l	30l	50m
1	41	51allophanic	100	25	1	80m	80h	100h
1	41	51allophanic	100	25	2	80m	80h	100h
1	42	51allophanic	80	25	1	80m	100h	100h
1	42	51allophanic	80	25	2	80m	100h	100h
1	43	51allophanic	60	25	1	60m	90h	100h
1	43	51allophanic	60	25	2	70m	60h	100h
1	44	51allophanic	40	25	1	20l	20l	40m
1	44	51allophanic	40	25	2	30l	30l	40m
1	45	21allophanic	100	25	1	20m	60h	100h
1	45	21allophanic	100	25	2	10l	80m	100h
1	46	21allophanic	80	25	1	70m	80h	100h
1	46	21allophanic	80	25	2	70m	100h	100h
1	47	21allophanic	60	25	1	90m	100m	100m
1	47	21allophanic	60	25	2	80m	80m	100m
1	48	21allophanic	40	25	1	30l	30l	50m
1	48	21allophanic	40	25	2	40l	40l	50m

1	49	45peat	100	25	1	30m	100h		100h	
1	49	45peat	100	25	2	40m	80h		100h	
1	50	45peat	80	25	1	30m	70h		100h	
1	50	45peat	80	25	2	30m	80h		100h	
1	51	45peat	60	25	1	20l	-1	-1	-1	-1
1	51	45peat	60	25	2	60m	60m		100h	
1	52	45peat	40	25	1	70l	80l		90m	
1	52	45peat	40	25	2	80l	80l		80m	
1	53	51peat	100	25	1	60m	90h		100h	
1	53	51peat	100	25	2	60l	90m		100h	
1	54	51peat	80	25	1	50m	90h		100h	
1	54	51peat	80	25	2	50m	90h		100h	
1	55	51peat	60	25	1	80m	100h		100h	
1	55	51peat	60	25	2	90m	100h		100h	
1	56	51peat	40	25	1	70l	80l		80m	
1	56	51peat	40	25	2	70l	70l		80m	
1	57	21peat	100	25	1	40m	100h		100h	
1	57	21peat	100	25	2	40m	80h		100h	
1	58	21peat	80	25	1	50m	100m		100h	
1	58	21peat	80	25	2	30m	80m		100h	
1	59	21peat	60	25	1	30l	100m		100h	
1	59	21peat	60	25	2	20l	100m		100h	
1	60	21peat	40	25	1	60l	50l		70m	
1	60	21peat	40	25	2	80l	80l		80m	
2	1	45pumice	100	25	1	80m	100m		100m	
2	1	45pumice	100	25	2	80m	100m		100h	
2	2	45pumice	80	25	1	70l	100h		100m	
2	2	45pumice	80	25	2	60l	100h		100m	
2	3	45pumice	60	25	1	40m	100m		100m	
2	3	45pumice	60	25	2	50m	100m		100m	
2	4	45pumice	40	25	1	60l	100h		100h	
2	4	45pumice	40	25	2	70m	100h		100h	
2	5	51pumice	100	25	1	50m	100m		100m	
2	5	51pumice	100	25	2	50m	100m		100h	
2	6	51pumice	80	25	1	40l	100h		100m	
2	6	51pumice	80	25	2	70m	100h		100m	
2	7	51pumice	60	25	1	90m	100m		100m	
2	7	51pumice	60	25	2	90m	100m		100m	
2	8	51pumice	40	25	1	80l	100h		100m	
2	8	51pumice	40	25	2	90l	100h		100m	
2	9	21pumice	100	25	1	60m	100l		100m	
2	9	21pumice	100	25	2	70m	100l		100l	
2	10	21pumice	80	25	1	50m	100m		100m	
2	10	21pumice	80	25	2	40l	100m		100m	
2	11	21pumice	60	25	1	50m	100m		100l	
2	11	21pumice	60	25	2	60m	100m		100l	
2	12	21pumice	40	25	1	50m	100m		100h	
2	12	21pumice	40	25	2	50m	100m		100l	
2	13	45ultic	100	25	1	100h	100h		100m	
2	13	45ultic	100	25	2	70h	100h		100h	
2	14	45ultic	80	25	1	80h	100m		100m	
2	14	45ultic	80	25	2	90h	100m		100m	
2	15	45ultic	60	25	1	90h	100m		100m	
2	15	45ultic	60	25	2	100h	100m		100m	

2	16	45ultic	40	25	1	30m	100l	100l
2	16	45ultic	40	25	2	30m	100l	100l
2	17	51ultic	100	25	1	100m	100h	100m
2	17	51ultic	100	25	2	100m	100h	100m
2	18	51ultic	80	25	1	100m	100h	100m
2	18	51ultic	80	25	2	100m	100h	100m
2	19	51ultic	60	25	1	100m	100h	100m
2	19	51ultic	60	25	2	100m	100h	100m
2	20	51ultic	40	25	1	70m	100l	100l
2	20	51ultic	40	25	2	70m	100l	100l
2	21	21ultic	100	25	1	60m	100h	100h
2	21	21ultic	100	25	2	90m	100h	100m
2	22	21ultic	80	25	1	100m	100m	100h
2	22	21ultic	80	25	2	80m	100m	100h
2	23	21ultic	60	25	1	80m	100m	100h
2	23	21ultic	60	25	2	90m	100m	100m
2	24	21ultic	40	25	1	40l	100l	100m
2	24	21ultic	40	25	2	40m	100l	100m
2	25	45granular	100	25	1	70m	100m	100m
2	25	45granular	100	25	2	90m	100h	100h
2	26	45granular	80	25	1	90h	100h	100m
2	26	45granular	80	25	2	90h	100h	100m
2	27	45granular	60	25	1	40h	100h	100m
2	27	45granular	60	25	2	80m	100h	100m
2	28	45granular	40	25	1	90m	100m	100m
2	28	45granular	40	25	2	80m	100h	100m
2	29	51granular	100	25	1	90m	100m	100m
2	29	51granular	100	25	2	100m	100m	100m
2	30	51granular	80	25	1	100m	100m	100m
2	30	51granular	80	25	2	100m	100m	100m
2	31	51granular	60	25	1	100m	100m	100m
2	31	51granular	60	25	2	80m	100m	100m
2	32	51granular	40	25	1	100m	100l	100m
2	32	51granular	40	25	2	90m	100l	100m
2	33	21granular	100	25	1	80m	100m	100m
2	33	21granular	100	25	2	80m	100m	100m
2	34	21granular	80	25	1	80m	100h	100h
2	34	21granular	80	25	2	90m	100h	100h
2	35	21granular	60	25	1	90m	100m	100h
2	35	21granular	60	25	2	100m	100m	100h
2	36	21granular	40	25	1	90m	100l	100m
2	36	21granular	40	25	2	80m	100m	100m
2	37	45allophanic	100	25	1	30l	90l	100m
2	37	45allophanic	100	25	2	20m	70l	80m
2	38	45allophanic	80	25	1	30l	90m	100m
2	38	45allophanic	80	25	2	30m	90m	100m
2	39	45allophanic	60	25	1	20l	80l	100m
2	39	45allophanic	60	25	2	30m	80l	100m
2	40	45allophanic	40	25	1	40l	90l	100m
2	40	45allophanic	40	25	2	30m	100m	100m
2	41	51allophanic	100	25	1	30l	50l	50m
2	41	51allophanic	100	25	2	60m	80m	100m
2	42	51allophanic	80	25	1	50l	100m	100m
2	42	51allophanic	80	25	2	40l	100m	100m

2	43	51allophanic	60	25	1	30m	100h		100m	
2	43	51allophanic	60	25	2	60m	100h		100m	
2	44	51allophanic	40	25	1	90l	100l		100l	
2	44	51allophanic	40	25	2	90m	100l		100l	
2	45	21allophanic	100	25	1	20l	100l		100m	
2	45	21allophanic	100	25	2	20l	100l		100m	
2	46	21allophanic	80	25	1	40l	100m		100m	
2	46	21allophanic	80	25	2	40l	100m		100m	
2	47	21allophanic	60	25	1	20l	100m		100m	
2	47	21allophanic	60	25	2	30m	100m		100m	
2	48	21allophanic	40	25	1	60m	100l		100m	
2	48	21allophanic	40	25	2	60m	100l		100m	
2	49	45peat	100	25	1	60m	100m		100m	
2	49	45peat	100	25	2	40m	100m		100m	
2	50	45peat	80	25	1	50m	100h		100h	
2	50	45peat	80	25	2	50m	100h		100h	
2	51	45peat	60	25	1	30m	100h		100h	
2	51	45peat	60	25	2	30m	100h		100h	
2	52	45peat	40	25	1	40m	90h		100h	
2	52	45peat	40	25	2	40m	90h		100h	
2	53	51peat	100	25	1	50l	100m		100m	
2	53	51peat	100	25	2	50m	100m		100m	
2	54	51peat	80	25	1	70m	100h		100h	
2	54	51peat	80	25	2	50m	100h		100h	
2	55	51peat	60	25	1	50m	100h		100h	
2	55	51peat	60	25	2	40m	100h		100h	
2	56	51peat	40	25	1	80m	100h		100m	
2	56	51peat	40	25	2	70m	100h		100m	
2	57	21peat	100	25	1	50m	100m		100h	
2	57	21peat	100	25	2	50m	100m		100h	
2	58	21peat	80	25	1	50m	100h		100h	
2	58	21peat	80	25	2	50m	100h		100h	
2	59	21peat	60	25	1	50m	100m		100h	
2	59	21peat	60	25	2	50m	100m		100h	
2	60	21peat	40	25	1	70m	100m		100h	
2	60	21peat	40	25	2	60m	100m		100h	
3	1	45waimak	100	25	1	100m	100h		-1	-1
3	1	45waimak	100	25	2	100m	100h		-1	-1
3	2	45waimak	80	25	1	100l	100m		-1	-1
3	2	45waimak	80	25	2	100l	100m		-1	-1
3	3	45waimak	60	25	1	90m	100h		-1	-1
3	3	45waimak	60	25	2	80m	100h		-1	-1
3	4	45waimak	40	25	1	90l	100h		-1	-1
3	4	45waimak	40	25	2	70l	100m		-1	-1
3	5	51waimak	100	25	1	100l	-1	-1	-1	-1
3	5	51waimak	100	25	2	100h	100h		-1	-1
3	6	51waimak	80	25	1	70m	100h		-1	-1
3	6	51waimak	80	25	2	90m	100h		-1	-1
3	7	51waimak	60	25	1	80m	100h		-1	-1
3	7	51waimak	60	25	2	40l	100m		-1	-1
3	8	51waimak	40	25	1	90l	100m		-1	-1
3	8	51waimak	40	25	2	90l	100m		-1	-1
3	9	21waimak	100	25	1	100h	100m		-1	-1
3	9	21waimak	100	25	2	100h	100m		-1	-1

3	10	21waimak	80	25	1	90m	100h		-1	-1
3	10	21waimak	80	25	2	90l	100h		-1	-1
3	11	21waimak	60	25	1	80l	100h		-1	-1
3	11	21waimak	60	25	2	90l	100h		-1	-1
3	12	21waimak	40	25	1	80l	100m		-1	-1
3	12	21waimak	40	25	2	90l	100m		-1	-1
3	13	45richmond	100	25	1	100m	100h		-1	-1
3	13	45richmond	100	25	2	100m	100h		-1	-1
3	14	45richmond	80	25	1	100m	100h		-1	-1
3	14	45richmond	80	25	2	100m	100h		-1	-1
3	15	45richmond	60	25	1	100m	100h		-1	-1
3	15	45richmond	60	25	2	100m	100h		-1	-1
3	16	45richmond	40	25	1	100m	100m		-1	-1
3	16	45richmond	40	25	2	100l	100m		-1	-1
3	17	51richmond	100	25	1	100h	100h		-1	-1
3	17	51richmond	100	25	2	100h	100h		-1	-1
3	18	51richmond	80	25	1	100h	100m		-1	-1
3	18	51richmond	80	25	2	100h	100m		-1	-1
3	19	51richmond	60	25	1	100h	100m		-1	-1
3	19	51richmond	60	25	2	90h	100m		-1	-1
3	20	51richmond	40	25	1	90m	100m		-1	-1
3	20	51richmond	40	25	2	90m	100m		-1	-1
3	21	21richmond	100	25	1	100m	100h		-1	-1
3	21	21richmond	100	25	2	100m	100h		-1	-1
3	22	21richmond	80	25	1	100m	100h		-1	-1
3	22	21richmond	80	25	2	100m	100h		-1	-1
3	23	21richmond	60	25	1	100l	100h		-1	-1
3	23	21richmond	60	25	2	100l	100h		-1	-1
3	24	21richmond	40	25	1	100l	100m		-1	-1
3	24	21richmond	40	25	2	100l	100m		-1	-1
3	25	45cass	100	25	1	70l	100m		-1	-1
3	25	45cass	100	25	2	60l	100m		-1	-1
3	26	45cass	80	25	1	50l	100m		-1	-1
3	26	45cass	80	25	2	50l	100m		-1	-1
3	27	45cass	60	25	1	60l	100m		-1	-1
3	27	45cass	60	25	2	70l	100m		-1	-1
3	28	45cass	40	25	1	70l	100m		-1	-1
3	28	45cass	40	25	2	60l	100m		-1	-1
3	29	51cass	100	25	1	70l	100m		-1	-1
3	29	51cass	100	25	2	70l	100m		-1	-1
3	30	51cass	80	25	1	70l	100m		-1	-1
3	30	51cass	80	25	2	80l	100m		-1	-1
3	31	51cass	60	25	1	80l	-1	-1	-1	-1
3	31	51cass	60	25	2	70l	100m		-1	-1
3	32	51cass	40	25	1	80l	100m		-1	-1
3	32	51cass	40	25	2	80l	100m		-1	-1
3	33	21cass	100	25	1	60l	100m		-1	-1
3	33	21cass	100	25	2	60l	100m		-1	-1
3	34	21cass	80	25	1	60l	100m		-1	-1
3	34	21cass	80	25	2	50l	100m		-1	-1
3	35	21cass	60	25	1	60l	100m		-1	-1
3	35	21cass	60	25	2	50l	100l		-1	-1
3	36	21cass	40	25	1	70l	100m		-1	-1
3	36	21cass	40	25	2	60l	100m		-1	-1

3	37	45broken river	100	25	1	5l	5m		-1	-1
3	37	45broken river	100	25	2	5l	5m		-1	-1
3	38	45broken river	80	25	1	20l	20m		-1	-1
3	38	45broken river	80	25	2	5l	5m		-1	-1
3	39	45broken river	60	25	1	10l	10m		-1	-1
3	39	45broken river	60	25	2	5l	5l		-1	-1
3	40	45broken river	40	25	1	10l	20l		-1	-1
3	40	45broken river	40	25	2	10l	20l		-1	-1
3	41	51broken river	100	25	1	10l	50l		-1	-1
3	41	51broken river	100	25	2	20l	50l		-1	-1
3	42	51broken river	80	25	1	10l	60m		-1	-1
3	42	51broken river	80	25	2	10l	30m		-1	-1
3	43	51broken river	60	25	1	20l	-1	-1	-1	-1
3	43	51broken river	60	25	2	5l	5m		-1	-1
3	44	51broken river	40	25	1	20l	50l		-1	-1
3	44	51broken river	40	25	2	20l	40l		-1	-1
3	45	21broken river	100	25	1	10l	10m		-1	-1
3	45	21broken river	100	25	2	20l	20m		-1	-1
3	46	21broken river	80	25	1	10l	30m		-1	-1
3	46	21broken river	80	25	2	10l	10m		-1	-1
3	47	21broken river	60	25	1	10l	10l		-1	-1
3	47	21broken river	60	25	2	10l	20m		-1	-1
3	48	21broken river	40	25	1	40l	20l		-1	-1
3	48	21broken river	40	25	2	40l	20l		-1	-1
3	49	45duncan	100	25	1	100h	100h		-1	-1
3	49	45duncan	100	25	2	100h	100h		-1	-1
3	50	45duncan	80	25	1	100h	100h		-1	-1
3	50	45duncan	80	25	2	100h	100h		-1	-1
3	51	45duncan	60	25	1	100h	100h		-1	-1
3	51	45duncan	60	25	2	100h	100h		-1	-1
3	52	45duncan	52	25	1	100h	100h		-1	-1
3	52	45duncan	52	25	2	100h	100h		-1	-1
3	53	51duncan	100	25	1	100h	100h		-1	-1
3	53	51duncan	100	25	2	100h	100h		-1	-1
3	54	51duncan	80	25	1	100h	100h		-1	-1
3	54	51duncan	80	25	2	100h	100h		-1	-1
3	55	51duncan	60	25	1	100h	100h		-1	-1
3	55	51duncan	60	25	2	100h	100h		-1	-1
3	56	51duncan	52	25	1	100h	100h		-1	-1
3	56	51duncan	52	25	2	100h	100h		-1	-1
3	57	21duncan	100	25	1	10h	50h		-1	-1
3	57	21duncan	100	25	2	100h	100m		-1	-1
3	58	21duncan	80	25	1	100h	100h		-1	-1
3	58	21duncan	80	25	2	100h	100h		-1	-1
3	59	21duncan	60	25	1	100h	100m		-1	-1
3	59	21duncan	60	25	2	100m	100m		-1	-1
3	60	21duncan	52	25	1	100h	100m		-1	-1
3	60	21duncan	52	25	2	100h	100m		-1	-1
3	61	45templeton	100	25	1	100h	100h		-1	-1
3	61	45templeton	100	25	2	100h	100h		-1	-1
3	62	45templeton	80	25	1	100m	100m		-1	-1
3	62	45templeton	80	25	2	100m	100m		-1	-1
3	63	45templeton	60	25	1	40l	40l		-1	-1
3	63	45templeton	60	25	2	100m	100m		-1	-1

3	64	45templeton	22	25	1	90l	100m		-1	-1
3	64	45templeton	22	25	2	100l	100l		-1	-1
3	65	51templeton	100	25	1	100m	100h		-1	-1
3	65	51templeton	100	25	2	100m	100h		-1	-1
3	66	51templeton	80	25	1	100m	100h		-1	-1
3	66	51templeton	80	25	2	90m	100h		-1	-1
3	67	51templeton	60	25	1	100m	100m		-1	-1
3	67	51templeton	60	25	2	100l	100m		-1	-1
3	68	51templeton	22	25	1	90l	100m		-1	-1
3	68	51templeton	22	25	2	80l	90m		-1	-1
3	69	21templeton	100	25	1	100m	100h		-1	-1
3	69	21templeton	100	25	2	100m	100h		-1	-1
3	70	21templeton	80	25	1	100l	100h		-1	-1
3	70	21templeton	80	25	2	100l	100h		-1	-1
3	71	21templeton	60	25	1	100l	100m		-1	-1
3	71	21templeton	60	25	2	100l	100m		-1	-1
3	72	21templeton	22	25	1	100l	100m		-1	-1
3	72	21templeton	22	25	2	90l	100l		-1	-1
4	1	45waimak	100	25	1	30m	50m		-1	-1
4	1	45waimak	100	25	2	90m	100h		-1	-1
4	2	45waimak	80	25	1	80m	80m		-1	-1
4	2	45waimak	80	25	2	80m	100m		-1	-1
4	3	45waimak	60	25	1	100m	80m		-1	-1
4	3	45waimak	60	25	2	70m	80m		-1	-1
4	4	45waimak	40	25	1	70m	100m		-1	-1
4	4	45waimak	40	25	2	80m	100m		-1	-1
4	5	51waimak	100	25	1	90m	100m		-1	-1
4	5	51waimak	100	25	2	90m	100m		-1	-1
4	6	51waimak	80	25	1	90m	100h		-1	-1
4	6	51waimak	80	25	2	80m	100h		-1	-1
4	7	51waimak	60	25	1	80m	100h		-1	-1
4	7	51waimak	60	25	2	80m	100h		-1	-1
4	8	51waimak	40	25	1	100m	100m		-1	-1
4	8	51waimak	40	25	2	70m	100m		-1	-1
4	9	21waimak	100	25	1	70m	100m		-1	-1
4	9	21waimak	100	25	2	90m	100h		-1	-1
4	10	21waimak	80	25	1	90m	100h		-1	-1
4	10	21waimak	80	25	2	80m	100h		-1	-1
4	11	21waimak	60	25	1	10l	30m		-1	-1
4	11	21waimak	60	25	2	5l	20m		-1	-1
4	12	21waimak	40	25	1	80l	100h		-1	-1
4	12	21waimak	40	25	2	80l	100h		-1	-1
4	13	45richmond	100	25	1	30m	20m		-1	-1
4	13	45richmond	100	25	2	20l	-1	-1	-1	-1
4	14	45richmond	80	25	1	60m	60m		-1	-1
4	14	45richmond	80	25	2	60m	60m		-1	-1
4	15	45richmond	60	25	1	50m	40m		-1	-1
4	15	45richmond	60	25	2	40m	50m		-1	-1
4	16	45richmond	40	25	1	80m	100m		-1	-1
4	16	45richmond	40	25	2	80m	100m		-1	-1
4	17	51richmond	100	25	1	50m	60m		-1	-1
4	17	51richmond	100	25	2	50m	100m		-1	-1
4	18	51richmond	80	25	1	50m	100h		-1	-1
4	18	51richmond	80	25	2	60m	100h		-1	-1

4	19	51richmond	60	25	1	80m	100h	-1	-1
4	19	51richmond	60	25	2	50m	100m	-1	-1
4	20	51richmond	40	25	1	100m	100h	-1	-1
4	20	51richmond	40	25	2	100m	100m	-1	-1
4	21	21richmond	100	25	1	40m	70m	-1	-1
4	21	21richmond	100	25	2	40m	60m	-1	-1
4	22	21richmond	80	25	1	70m	100h	-1	-1
4	22	21richmond	80	25	2	60m	100m	-1	-1
4	23	21richmond	60	25	1	60m	100m	-1	-1
4	23	21richmond	60	25	2	60m	80m	-1	-1
4	24	21richmond	40	25	1	90m	100m	-1	-1
4	24	21richmond	40	25	2	90m	100h	-1	-1
4	25	45cass	100	25	1	60m	60m	-1	-1
4	25	45cass	100	25	2	50m	50m	-1	-1
4	26	45cass	80	25	1	50m	30m	-1	-1
4	26	45cass	80	25	2	20l	20m	-1	-1
4	27	45cass	60	25	1	40m	30m	-1	-1
4	27	45cass	60	25	2	30m	40m	-1	-1
4	28	45cass	40	25	1	60m	60m	-1	-1
4	28	45cass	40	25	2	60m	60m	-1	-1
4	29	51cass	100	25	1	60m	70m	-1	-1
4	29	51cass	100	25	2	60m	70m	-1	-1
4	30	51cass	80	25	1	50m	50m	-1	-1
4	30	51cass	80	25	2	50m	50m	-1	-1
4	31	51cass	60	25	1	50m	100m	-1	-1
4	31	51cass	60	25	2	60m	100m	-1	-1
4	32	51cass	40	25	1	70m	100m	-1	-1
4	32	51cass	40	25	2	70m	90m	-1	-1
4	33	21cass	100	25	1	30m	40m	-1	-1
4	33	21cass	100	25	2	20l	30m	-1	-1
4	34	21cass	80	25	1	20m	20m	-1	-1
4	34	21cass	80	25	2	20m	30m	-1	-1
4	35	21cass	60	25	1	30m	60m	-1	-1
4	35	21cass	60	25	2	30m	40m	-1	-1
4	36	21cass	40	25	1	50m	60m	-1	-1
4	36	21cass	40	25	2	50m	90m	-1	-1
4	37	45broken river	100	25	1	5h	5m	-1	-1
4	37	45broken river	100	25	2	5h	5m	-1	-1
4	38	45broken river	80	25	1	5h	5m	-1	-1
4	38	45broken river	80	25	2	5h	5m	-1	-1
4	39	45broken river	60	25	1	5h	1h	-1	-1
4	39	45broken river	60	25	2	5h	5m	-1	-1
4	40	45broken river	40	25	1	5l	5l	-1	-1
4	40	45broken river	40	25	2	20l	10m	-1	-1
4	41	51broken river	100	25	1	30m	50m	-1	-1
4	41	51broken river	100	25	2	40m	50m	-1	-1
4	42	51broken river	80	25	1	20m	10m	-1	-1
4	42	51broken river	80	25	2	10m	10m	-1	-1
4	43	51broken river	60	25	1	10m	5l	-1	-1
4	43	51broken river	60	25	2	5m	5m	-1	-1
4	44	51broken river	40	25	1	5m	20m	-1	-1
4	44	51broken river	40	25	2	5m	20m	-1	-1
4	45	21broken river	100	25	1	10m	10m	-1	-1
4	45	21broken river	100	25	2	10m	20m	-1	-1

4	46	21broken river	80	25	1	10m	5m		-1	-1
4	46	21broken river	80	25	2	5m	5m		-1	-1
4	47	21broken river	60	25	1	5m	5m		-1	-1
4	47	21broken river	60	25	2	5m	5m		-1	-1
4	48	21broken river	40	25	1	5m	10m		-1	-1
4	48	21broken river	40	25	2	5m	10m		-1	-1
4	49	45duncan	100	25	1	100h	100h		-1	-1
4	49	45duncan	100	25	2	100h	100h		-1	-1
4	50	45duncan	80	25	1	100h	100h		-1	-1
4	50	45duncan	80	25	2	100h	100h		-1	-1
4	51	45duncan	60	25	1	100m	100h		-1	-1
4	51	45duncan	60	25	2	100m	100h		-1	-1
4	52	45duncan	40	25	1	100m	100h		-1	-1
4	52	45duncan	40	25	2	100m	100h		-1	-1
4	53	51duncan	100	25	1	100m	100h		-1	-1
4	53	51duncan	100	25	2	100m	100h		-1	-1
4	54	51duncan	80	25	1	100h	100h		-1	-1
4	54	51duncan	80	25	2	100h	100h		-1	-1
4	55	51duncan	60	25	1	100m	100h		-1	-1
4	55	51duncan	60	25	2	100m	100h		-1	-1
4	56	51duncan	40	25	1	100h	100h		-1	-1
4	56	51duncan	40	25	2	100h	100h		-1	-1
4	57	21duncan	100	25	1	100m	100h		-1	-1
4	57	21duncan	100	25	2	100m	100h		-1	-1
4	58	21duncan	80	25	1	100m	100h		-1	-1
4	58	21duncan	80	25	2	100m	100h		-1	-1
4	59	21duncan	60	25	1	100m	100h		-1	-1
4	59	21duncan	60	25	2	100m	100h		-1	-1
4	60	21duncan	40	25	1	100m	100h		-1	-1
4	60	21duncan	40	25	2	100m	100h		-1	-1
4	61	45templeton	100	25	1	100h	100h		-1	-1
4	61	45templeton	100	25	2	100h	100h		-1	-1
4	62	45templeton	80	25	1	100m	100h		-1	-1
4	62	45templeton	80	25	2	100m	100h		-1	-1
4	63	45templeton	60	25	1	100m	100h		-1	-1
4	63	45templeton	60	25	2	100m	100h		-1	-1
4	64	45templeton	40	25	1	100m	100h		-1	-1
4	64	45templeton	40	25	2	100m	100h		-1	-1
4	65	51templeton	100	25	1	100h	-1	-1	-1	-1
4	65	51templeton	100	25	2	100m	100h		-1	-1
4	66	51templeton	80	25	1	100m	100h		-1	-1
4	66	51templeton	80	25	2	100m	100h		-1	-1
4	67	51templeton	60	25	1	100m	100h		-1	-1
4	67	51templeton	60	25	2	100m	100h		-1	-1
4	68	51templeton	40	25	1	100m	100h		-1	-1
4	68	51templeton	40	25	2	100m	100h		-1	-1
4	69	21templeton	100	25	1	100h	100h		-1	-1
4	69	21templeton	100	25	2	100h	100h		-1	-1
4	70	21templeton	80	25	1	100m	100h		-1	-1
4	70	21templeton	80	25	2	100m	100h		-1	-1
4	71	21templeton	60	25	1	100m	100h		-1	-1
4	71	21templeton	60	25	2	100m	100h		-1	-1
4	72	21templeton	40	25	1	100m	100h		-1	-1
4	72	21templeton	40	25	2	100m	100h		-1	-1

Appendix to Chapter 7

Experiment 1: PCP residue data based on ANOVA

With wr = treatment consisting of
1 = autoclaved and soil control combined
2 = HR131
3 = HR160
4 = *P. sordida*

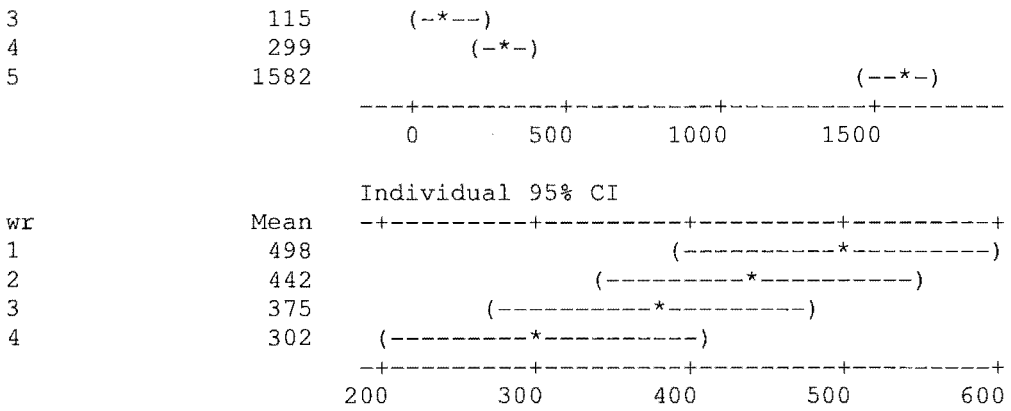
Two-way Analysis of Variance

logged values for final conc

```
Analysis of Variance for logged values (lv)
Source        DF        SS        MS        F        P
pcpconc        4   46.9280   11.7320   885.18    0.000
wr             3    0.5598    0.1866    14.08    0.000
Interaction   12    0.5324    0.0444     3.35    0.008
Error         20    0.2651    0.0133
Total         39   48.2853

                        Individual 95% CI
pcpconc         Mean    --+---------+---------+---------+---------
1              0.000     (*)
2              1.323                (-*)
3              2.021                      (*)
4              2.449                           (*)
5              3.189                                      (*)
                        --+---------+---------+---------+---------
                      0.000     0.800     1.600     2.400

                        Individual 95% CI
wr              Mean    ----+---------+---------+---------+-------
1              1.915                              (------*-----)
2              1.836                         (-----*-----)
3              1.835                         (-----*-----)
4              1.599     (-----*------)
                        ----+---------+---------+---------+-------
                        1.560     1.680     1.800     1.920
```

Two-way Analysis of Variance

```
Analysis of Variance for finconc (PCP not ln-transformed)
Source        DF        SS        MS        F        P
pcpconc        4  14319645   3579911   141.70    0.000
wr             3    214460     71487     2.83    0.065
Interaction   12    438882     36574     1.45    0.224
Error         20    505286     25264
Total         39  15478273

                        Individual 95% CI
pcpconc         Mean    ---+---------+---------+---------+--------
1                 -0     (-*-)
2                 24     (-*--)
```




```
Worksheet size: 100000 cells
Retrieving project from file: K:\BIOREMEDIATION\KIRSTY\PCP1
PAPER\PCP1RESIDUE ANALYSIS 2.MPJ
```

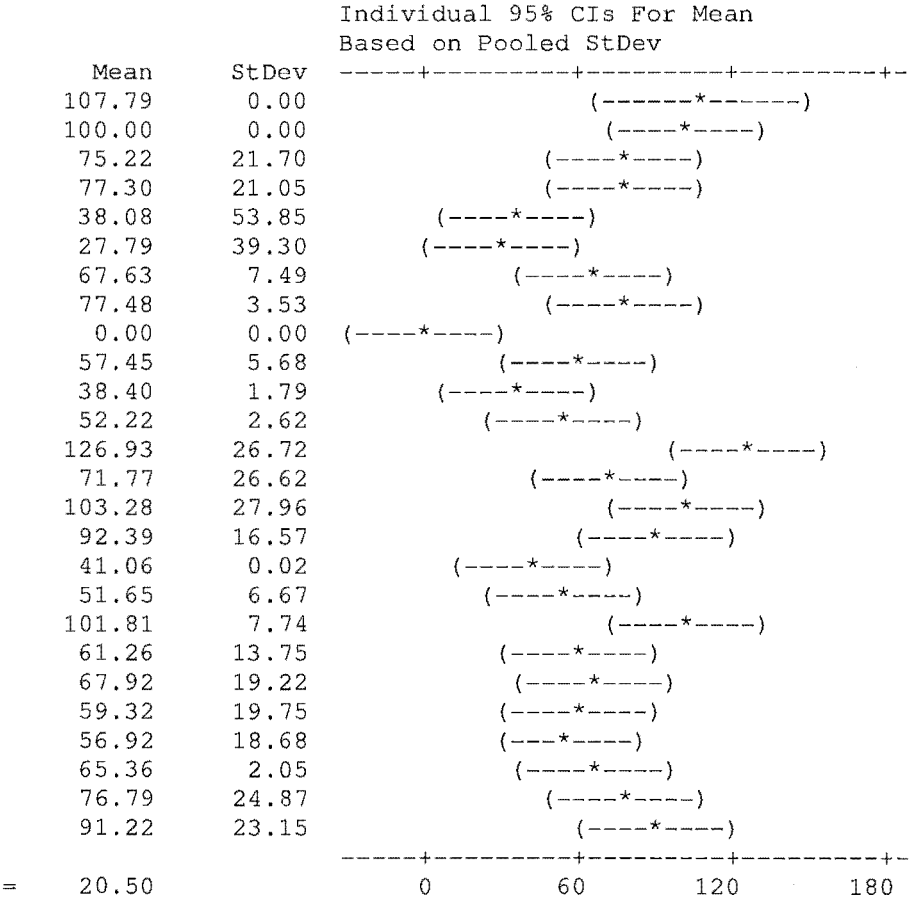
Experiment 2: PCP residue data and fungal growth data based on ANOVA

One-way Analysis of Variance

```
Analysis of Variance for PCP concentration
Source     DF        SS        MS        F        P
isolate    25     37493      1500     3.57    0.001
Error      25     10509       420
Total      50     48002
```

Level	N	Mean	StDev
0.00	1	107.79	0.00
1.00	2	100.00	0.00
2.00	2	75.22	21.70
12.00	2	77.30	21.05
21.00	2	38.08	53.85
36.00	2	27.79	39.30
43.00	2	67.63	7.49
45.00	2	77.48	3.53
51.00	2	0.00	0.00
83.00	2	57.45	5.68
87.00	2	38.40	1.79
88.00	2	52.22	2.62
100.02	2	126.93	26.72
100.11	2	71.77	26.62
100.16	2	103.28	27.96
100.17	2	92.39	16.57
114.00	2	41.06	0.02
128.00	2	51.65	6.67
144.03	2	101.81	7.74
149.00	2	61.26	13.75
152.00	2	67.92	19.22
160.00	2	59.32	19.75
244.00	2	56.92	18.68
375.00	2	65.36	2.05
388.00	2	76.79	24.87
389.00	2	91.22	23.15

Pooled StDev = 20.50




```
Fisher's pairwise comparisons

    Family error rate = 0.914
Individual error rate = 0.0500

Critical value = 2.060

Intervals for (column level mean) - (row level mean)

                0.00        1.00        2.00       12.00       21.00       36.00

    1.00       -43.9
                59.5
```

2.00	-19.2	-17.5				
	84.3	67.0				
12.00	-21.2	-19.5	-44.3			
	82.2	64.9	40.2			
21.00	18.0	19.7	-5.1	-3.0		
	121.4	104.2	79.4	81.5		
36.00	28.3	30.0	5.2	7.3	-31.9	
	131.7	114.4	89.7	91.7	52.5	
43.00	-11.6	-9.9	-34.6	-32.6	-71.8	-82.1
	91.9	74.6	49.8	51.9	12.7	2.4
45.00	-21.4	-19.7	-44.5	-42.4	-81.6	-91.9
	82.0	64.8	40.0	42.1	2.8	-7.5
51.00	56.1	57.8	33.0	35.1	-4.2	-14.4
	159.5	142.2	117.5	119.5	80.3	70.0
83.00	-1.4	0.3	-24.5	-22.4	-61.6	-71.9
	102.1	84.8	60.0	62.1	22.9	12.6
87.00	17.7	19.4	-5.4	-3.3	-42.6	-52.8
	121.1	103.8	79.1	81.1	41.9	31.6
88.00	3.8	5.5	-19.2	-17.2	-56.4	-66.7
	107.3	90.0	65.2	67.3	28.1	17.8
100.02	-70.9	-69.2	-93.9	-91.9	-131.1	-141.4
	32.6	15.3	-9.5	-7.4	-46.6	-56.9
100.11	-15.7	-14.0	-38.8	-36.7	-75.9	-86.2
	87.8	70.5	45.7	47.8	8.5	-1.7
100.16	-47.2	-45.5	-70.3	-68.2	-107.4	-117.7
	56.2	39.0	14.2	16.3	-23.0	-33.3
100.17	-36.3	-34.6	-59.4	-57.3	-96.5	-106.8
	67.1	49.8	25.1	27.1	-12.1	-22.4
114.00	15.0	16.7	-8.1	-6.0	-45.2	-55.5
	118.5	101.2	76.4	78.5	39.2	29.0
128.00	4.4	6.1	-18.7	-16.6	-55.8	-66.1
	107.9	90.6	65.8	67.9	28.7	18.4
144.03	-45.7	-44.0	-68.8	-66.7	-106.0	-116.3
	57.7	40.4	15.6	17.7	-21.5	-31.8
149.00	-5.2	-3.5	-28.3	-26.2	-65.4	-75.7
	98.3	81.0	56.2	58.3	19.1	8.8
152.00	-11.9	-10.2	-34.9	-32.9	-72.1	-82.4
	91.6	74.3	49.5	51.6	12.4	2.1
160.00	-3.3	-1.6	-26.3	-24.3	-63.5	-73.8
	100.2	82.9	58.1	60.2	21.0	10.7

244.00	-0.8	0.9	-23.9	-21.8	-61.1	-71.4
	102.6	85.3	60.5	62.6	23.4	13.1
375.00	-9.3	-7.6	-32.4	-30.3	-69.5	-79.8
	94.2	76.9	52.1	54.2	15.0	4.7
388.00	-20.7	-19.0	-43.8	-41.7	-80.9	-91.2
	82.7	65.4	40.7	42.7	3.5	-6.8
389.00	-35.2	-33.5	-58.2	-56.2	-95.4	-105.7
	68.3	51.0	26.2	28.3	-10.9	-21.2

	43.00	45.00	51.00	83.00	87.00	88.00
45.00	-52.1					
	32.4					
51.00	25.4	35.2				
	109.9	119.7				
83.00	-32.0	-22.2	-99.7			
	52.4	62.3	-15.2			
87.00	-13.0	-3.2	-80.6	-23.2		
	71.5	81.3	3.8	61.3		
88.00	-26.8	-17.0	-94.5	-37.0	-56.1	
	57.6	67.5	-10.0	47.5	28.4	
100.02	-101.5	-91.7	-169.2	-111.7	-130.8	-116.9
	-17.1	-7.2	-84.7	-27.2	-46.3	-32.5
100.11	-46.4	-36.5	-114.0	-56.6	-75.6	-61.8
	38.1	47.9	-29.5	27.9	8.9	22.7
100.16	-77.9	-68.0	-145.5	-88.1	-107.1	-93.3
	6.6	16.4	-61.0	-3.6	-22.6	-8.8
100.17	-67.0	-57.1	-134.6	-77.2	-96.2	-82.4
	17.5	27.3	-50.2	7.3	-11.8	2.1
114.00	-15.7	-5.8	-83.3	-25.9	-44.9	-31.1
	68.8	78.6	1.2	58.6	39.6	53.4
128.00	-26.3	-16.4	-93.9	-36.4	-55.5	-41.7
	58.2	68.1	-9.4	48.0	29.0	42.8
144.03	-76.4	-66.6	-144.0	-86.6	-105.6	-91.8
	8.1	17.9	-59.6	-2.1	-21.2	-7.4
149.00	-35.9	-26.0	-103.5	-46.0	-65.1	-51.3
	48.6	58.5	-19.0	38.4	19.4	33.2
152.00	-42.5	-32.7	-110.2	-52.7	-71.8	-57.9
	41.9	51.8	-25.7	31.8	12.7	26.5
160.00	-33.9	-24.1	-101.6	-44.1	-63.2	-49.3
	50.6	60.4	-17.1	40.4	21.3	35.1
244.00	-31.5	-21.7	-99.1	-41.7	-60.8	-46.9

	53.0	62.8	-14.7	42.8	23.7	37.5
375.00	-40.0	-30.1	-107.6	-50.1	-69.2	-55.4
	44.5	54.4	-23.1	34.3	15.3	29.1
388.00	-51.4	-41.5	-119.0	-61.6	-80.6	-66.8
	33.1	42.9	-34.6	22.9	3.8	17.7
389.00	-65.8	-56.0	-133.5	-76.0	-95.1	-81.2
	18.6	28.5	-49.0	8.5	-10.6	3.2

	100.02	100.11	100.16	100.17	114.00	128.00
100.11	12.9					
	97.4					
100.16	-18.6	-73.7				
	65.9	10.7				
100.17	-7.7	-62.9	-31.3			
	76.8	21.6	53.1			
114.00	43.6	-11.5	20.0	9.1		
	128.1	72.9	104.4	93.6		
128.00	33.0	-22.1	9.4	-1.5	-52.8	
	117.5	62.3	93.9	83.0	31.6	
144.03	-17.1	-72.3	-40.8	-51.7	-103.0	-92.4
	67.4	12.2	43.7	32.8	-18.5	-7.9
149.00	23.4	-31.7	-0.2	-11.1	-62.4	-51.8
	107.9	52.7	84.3	73.4	22.0	32.6
152.00	16.8	-38.4	-6.9	-17.8	-69.1	-58.5
	101.2	46.1	77.6	66.7	15.4	26.0
160.00	25.4	-29.8	1.7	-9.2	-60.5	-49.9
	109.8	54.7	86.2	75.3	24.0	34.6
244.00	27.8	-27.4	4.1	-6.8	-58.1	-47.5
	112.2	57.1	88.6	77.7	26.4	37.0
375.00	19.3	-35.8	-4.3	-15.2	-66.5	-55.9
	103.8	48.6	80.2	69.3	17.9	28.5
388.00	7.9	-47.3	-15.7	-26.6	-78.0	-67.4
	92.4	37.2	68.7	57.8	6.5	17.1
389.00	-6.5	-61.7	-30.2	-41.1	-92.4	-81.8
	77.9	22.8	54.3	43.4	-7.9	2.7

	144.03	149.00	152.00	160.00	244.00	375.00
149.00	-1.7					
	82.8					
152.00	-8.3	-48.9				
	76.1	35.6				

160.00	0.3	-40.3	-33.6			
	84.7	44.2	50.8			
244.00	2.7	-37.9	-31.2	-39.8		
	87.1	46.6	53.2	44.6		
375.00	-5.8	-46.3	-39.7	-48.3	-50.7	
	78.7	38.1	44.8	36.2	33.8	
388.00	-17.2	-57.8	-51.1	-59.7	-62.1	-53.7
	67.3	26.7	33.4	24.8	22.4	30.8
389.00	-31.6	-72.2	-65.5	-74.1	-76.5	-68.1
	52.8	12.3	18.9	10.3	7.9	16.4

	388.00
389.00	-56.7
	27.8

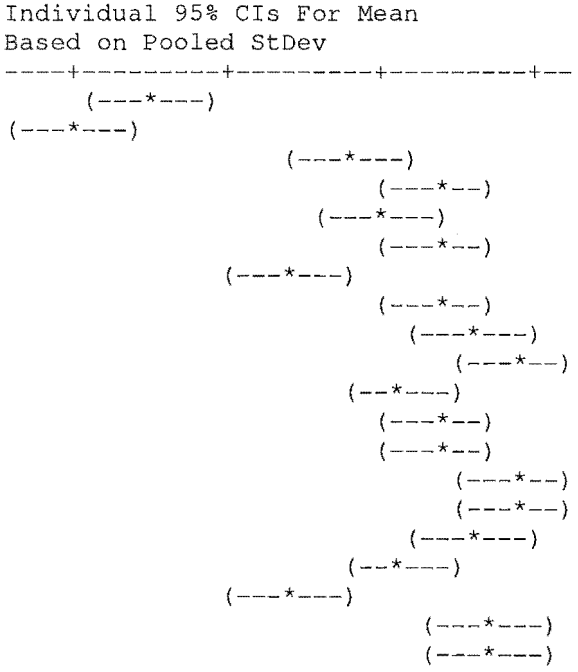
```
Worksheet size: 100000 cells
Retrieving project from file: K:\Bioremediation\kirsty\PCP 2 or isolate
screen 1\isolate screen 1a.MPJ
```

growth at 1 wk

One-way Analysis of Variance

```
Analysis of Variance for growth1w
Source     DF        SS        MS        F        P
isolate    25   39988.9    1599.6    19.23    0.000
Error      26    2162.5      83.2
Total      51   42151.4
```

Level	N	Mean	StDev
0.00	2	17.50	10.61
1.00	2	0.00	0.00
2.00	2	62.50	17.68
12.00	2	82.50	10.61
21.00	2	70.00	28.28
36.00	2	82.50	10.61
43.00	2	50.00	0.00
45.00	2	82.50	10.61
51.00	2	90.00	0.00
83.00	2	100.00	0.00
87.00	2	75.00	0.00
88.00	2	82.50	10.61
100.02	2	82.50	10.61
100.11	2	100.00	0.00
100.16	2	100.00	0.00
100.17	2	90.00	0.00
114.00	2	75.00	0.00
128.00	2	50.00	0.00
144.03	2	95.00	7.07
149.00	2	95.00	7.07



```
152.00       2      82.50      10.61                                (---*--)
160.00       2      75.00       0.00                              (--*---)
244.00       2      95.00       7.07                                   (---*---)
375.00       2      90.00       0.00                                 (---*---)
388.00       2      25.00       0.00          (---*---)
389.00       2      17.50      10.61        (---*---)
                                        -----+---------+---------+---------+--
Pooled StDev =      9.12                    0        35        70        105
```

Correlations (Pearson)

```
Correlation of PCP concentration and growth1wk = -0.259, P-Value = 0.066
```

Regression Analysis

```
The regression equation is
PCP concentration = 88.6 - 0.284 growth1wk

51 cases used 1 cases contain missing values

Predictor        Coef       StDev          T        P
Constant        88.64       11.79       7.52    0.000
growth1w      -0.2842      0.1513      -1.88    0.066

S = 30.23       R-Sq = 6.7%      R-Sq(adj) = 4.8%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         1      3224.8      3224.8      3.53    0.066
Residual Error    49     44776.9       913.8
Total             50     48001.6

Unusual Observations
Obs   growth1w    percont         Fit   StDev Fit    Residual    St Resid
  5         90       0.00       63.06        4.97      -63.06       -2.11R
  8         75       0.00       67.32        4.25      -67.32       -2.25R
 13         90       0.00       63.06        4.97      -63.06       -2.11R
 14         90       0.00       63.06        4.97      -63.06       -2.11R
 35         10     107.79       85.80       10.39       21.99        0.77 X
 40         10     107.59       85.80       10.39       21.79        0.77 X
 41         75     145.82       67.32        4.25       78.50        2.62R
 45        100     123.05       60.22        5.91       62.83        2.12R
 51          0     100.00       88.64       11.79       11.36        0.41 X
 52          0     100.00       88.64       11.79       11.36        0.41 X

R denotes an observation with a large standardized residual
X denotes an observation whose X value gives it large influence.


Worksheet size: 100000 cells
Retrieving project from file: K:\BIOREMEDIATION\KIRSTY\SCREENING\ISOLATE
SCREEN 1B.MPJ
```

Regression Analysis

```
The regression equation is
PCP concentration = 88.6 - 0.284 growth1wk

51 cases used 1 cases contain missing values

Predictor        Coef       StDev          T        P
Constant        88.64       11.79       7.52    0.000
growth1w      -0.2842      0.1513      -1.88    0.066

S = 30.23       R-Sq = 6.7%      R-Sq(adj) = 4.8%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         1      3224.8      3224.8      3.53    0.066
Residual Error    49     44776.9       913.8
Total             50     48001.6

Unusual Observations
Obs   growth1w    percont         Fit   StDev Fit    Residual    St Resid
  5         90       0.00       63.06        4.97      -63.06       -2.11R
  8         75       0.00       67.32        4.25      -67.32       -2.25R
 13         90       0.00       63.06        4.97      -63.06       -2.11R
 14         90       0.00       63.06        4.97      -63.06       -2.11R
 35         10     107.79       85.80       10.39       21.99        0.77 X
 40         10     107.59       85.80       10.39       21.79        0.77 X
 41         75     145.82       67.32        4.25       78.50        2.62R
 45        100     123.05       60.22        5.91       62.83        2.12R
 51          0     100.00       88.64       11.79       11.36        0.41 X
 52          0     100.00       88.64       11.79       11.36        0.41 X

R denotes an observation with a large standardized residual
X denotes an observation whose X value gives it large influence.
```

Regression Analysis

```
The regression equation is
percont = 95.3 - 0.343 growth2wks

51 cases used 1 cases contain missing values

Predictor        Coef       StDev          T        P
Constant        95.26       13.19       7.22    0.000
growth2w      -0.3433      0.1574      -2.18    0.034

S = 29.88       R-Sq = 8.9%      R-Sq(adj) = 7.0%

Analysis of Variance

Source            DF          SS          MS         F        P
Regression         1      4249.1      4249.1      4.76    0.034
Residual Error    49     43752.5       892.9
Total             50     48001.6

Unusual Observations
```

Obs	growth2w	percont	Fit	StDev Fit	Residual	St Resid
5	90	0.00	64.37	4.50	-64.37	-2.18R
8	75	0.00	69.51	4.24	-69.51	-2.35R
13	90	0.00	64.37	4.50	-64.37	-2.18R
14	100	0.00	60.93	5.28	-60.93	-2.07R
35	10	107.79	91.83	11.71	15.97	0.58 X
40	10	107.59	91.83	11.71	15.76	0.57 X
41	75	145.82	69.51	4.24	76.31	2.58R
45	100	123.05	60.93	5.28	62.12	2.11R
51	0	100.00	95.26	13.19	4.74	0.18 X
52	0	100.00	95.26	13.19	4.74	0.18 X

R denotes an observation with a large standardized residual
X denotes an observation whose X value gives it large influence.

Experiment 5: PCP residue and fungal growth data

PCP residue (mg/kg) and fungal colonisation data. Note: in the later part composite samples (2 reps/composite sample) were analysed

treatment	isolate	rep	growth h8	growth h16	growth h30	growth h42	growth h84	growth h126	growth h168	residue day0	residue day42	residue day84	Residue day126 1st analysis	Residue day126 2nd analysis
3	HR112	1	1	0	0	0	0	0	0		537	622	399	193
3	HR112	2	0	0	0	0	5	0	1		705		353	177
3	HR112	3	0	0	0	0	0	0	0		1016			
3	HR112	4	0	0	0	0	0	0	0		734			
3	HR112	5	0	0	0	0	0	0	0		865	706		
7	HR122	1	10	10	10	0	0	25	25		625	418	751	530
7	HR122	2	10	10	10	10	5	0	1		532		563	988
7	HR122	3	5	5	25	25	5	0	0		537			
7	HR122	4	5	5	0	0	1	0	0		539			
7	HR122	5	5	10	25	25	10	0	0		565	538		
11	HR131	1	1	1	5	5	5	5	5		853		554	615
11	HR131	2	1	0	5	0	0	0	0		735		535	589
11	HR131	3	0	1	0	0	0	0	0		762			
11	HR131	4	0	1	0	0	0	0	0		635	831		
11	HR131	5	0	0	1	5	5	5	5		670	645		
15	HR160	1	10	5	5	10	10	0	5		921	639	296	100
15	HR160	2	5	5	5	5	5	5	1		746		271	70
15	HR160	3	5	5	5	0	0	0	0		638	212		
15	HR160	4	10	25	5	0	0	10	5		695			
15	HR160	5	25	25	10	0	0	5	0		557			
19	HR577	1	1	1	5	0	0	0	10		845		714	826
19	HR577	2	1	1	0	10	10	25	0		880		739	831
19	HR577	3	0	1	5	0	0	0	0		878			
19	HR577	4	1	1	5	10	10	20	10		746	1282		
19	HR577	5	1	5	0	10	10	20	10		417	966		
23	PS	1	1	5	5	0	0	0	0		794		257	114
23	PS	2	1	5	25	25	50	50	50		261	185	244	123
23	PS	3	5	1	1	0	0	0	0		418	141		
23	PS	4	5	1	1	0	0	0	0		180			

23	PS sterile	5	5	10	1	0	0	0	0		251			
27	scs sterile	1	0	0	0	0	0	0	0	1168	777		458	378
27	scs sterile	2	0	0	0	0	0	0	0	1149	1429	279	496	242
27	scs sterile	3	0	0	0	0	0	0	0	930	764			
27	scs sterile	4	0	0	0	0	0	0	0	1153	808			
27	scs	5	0	0	0	0	0	0	0	1188	866	274		

Appendix to Chapter 8

PCP residue data

GCMS Analysis

Submitters Ref	Sampling Date	Lab Ref	PCP (ppm in dry soil)	PCA (ppm in dry soil)
Soil Cell 2a	28-Jan-00	W0067-1A	362	1
Soil Cell 2a	28-Jan-00	W0067-1B	250	1
Soil Cell 1	28-Jan-00	W0067-2A	1597	2
Soil Cell 1	28-Jan-00	W0067-2B	1135	1
Soil Cell 2	28-Jan-00	W0067-3A	1772	1
Soil Cell 2	28-Jan-00	W0067-3B	422	216
Soil Cell 2a	11-Feb-00	W0075-1A	1085	0
Soil Cell 2a	11-Feb-00	W0075-1B	1248	0
Soil Cell 1	11-Feb-00	W0075-2A	470	0
Soil Cell 1	11-Feb-00	W0075-2B	571	0
Soil Cell 2	11-Feb-00	W0075-3A	691	0
Soil Cell 2	11-Feb-00	W0075-3B	435	0
Soil Cell 2a	25-Feb-00	W0077-4A	502	0
Soil Cell 2a	25-Feb-00	W0077-4B	100	0
Soil Cell 1	25-Feb-00	W0077-5A	827	0
Soil Cell 1	25-Feb-00	W0077-5B	319	1
Soil Cell 2	25-Feb-00	W0077-6A	94	0
Soil Cell 2	25-Feb-00	W0077-6B	263	0
Soil Cell 2a	11-Mar-00	W0086-7A	179	0
Soil Cell 2a	11-Mar-00	W0086-7B	116	0
Soil Cell 1	11-Mar-00	W0086-8A	551	0
Soil Cell 1	11-Mar-00	W0086-8B	1071	0
Soil Cell 2	11-Mar-00	W0086-9A	417	0
Soil Cell 2	11-Mar-00	W0086-9B	135	0
Soil Cell 4	24-Mar-00	W0088-1A	747	0
Soil Cell 4	24-Mar-00	W0088-1B	698	0
Soil Cell 3	24-Mar-00	W0088-2A	304	0
Soil Cell 3	24-Mar-00	W0088-2B	423	0
Soil Cell 2a	7-Apr-00	W0097-1	31	0

Soil Cell 2a	7-Apr-00	W0097-2	130	0
Soil Cell 1	7-Apr-00	W0097-3	281	0
Soil Cell 1	7-Apr-00	W0097-4	221	0
Soil Cell 2	7-Apr-00	W0097-5	90	0
Soil Cell 2	7-Apr-00	W0097-6	80	0
Soil Cell 4	14-Apr-00	W0098-7	456	5
Soil Cell 4	14-Apr-00	W0098-8	271	5
Soil Cell 3	14-Apr-00	W0098-9	139	1
Soil Cell 3	14-Apr-00	W0098-10	114	1
Soil Cell 4	31-Mar-00	W0103-11	757	2
Soil Cell 4	31-Mar-00	W0103-12	255	2
Soil Cell 3	31-Mar-00	W0103-13	130	1
Soil Cell 3	31-Mar-00	W0103-14	151	1
Soil Cell 4	21-Apr-00	W0104-15	841	4
Soil Cell 4	21-Apr-00	W0104-16	678	4
Soil Cell 3	21-Apr-00	W0104-17	80	0
Soil Cell 3	21-Apr-00	W0104-18	136	0
Soil Cell 2a	30-May-00	W0117-19	363	0
Soil Cell 2a	30-May-00	W0117-20	88	0
Soil Cell 1	30-May-00	W0117-21	774	1
Soil Cell 1	30-May-00	W0117-22	758	0
Soil Cell 2	30-May-00	W0117-23	1	0
Soil Cell 2	30-May-00	W0117-24	0	0
Soil Cell 3	30-May-00	W0117-25	322	1
Soil Cell 3	30-May-00	W0117-26	98	1
Soil Cell 4	30-May-00	W0117-27	486	4
Soil Cell 4	30-May-00	W0117-28	854	4
Soil Cell 2a	9-Jun-00	W0124-1	353	0
Soil Cell 2a	9-Jun-00	W0124-2	159	0
Soil Cell 2a	9-Jun-00	W0124-3	226	0
Soil Cell 2a	9-Jun-00	W0124-4	253	0
Soil Cell 2a	9-Jun-00	W0124-5	205	0
Soil Cell 1	9-Jun-00	W0124-6	65	0
Soil Cell 1	9-Jun-00	W0124-7	448	0
Soil Cell 1	9-Jun-00	W0124-8	48	0
Soil Cell 1	9-Jun-00	W0124-9	81	0
Soil Cell 1	9-Jun-00	W0124-10	106	0
Soil Cell 2	9-Jun-00	W0124-11	164	0
Soil Cell 2	9-Jun-00	W0124-12	103	0

Soil Cell 2	9-Jun-00	W0124-13	138	0
Soil Cell 2	9-Jun-00	W0124-14	58	0
Soil Cell 2	9-Jun-00	W0124-15	79	0
Soil Cell 2	9-Jun-00	W0124-16	69	0
Emptied soil cells Into skip	Nov 01			
Skip1	Nov 01	Mwr1	120	Not tested
Skip2	Nov 01	Mwr2	10	Not tested
Skip3	Nov 01	Mwr3	0	Not tested
Skip4	Nov 01	Mwr4	51	Not tested
Skip5	Nov 01	Mwr5	35	Not tested
Skip1	Dec 01	RMW327	0	Not tested
Skip2	Dec 01	RMW328	58	Not tested
Skip3	Dec 01	RMW329	63	Not tested
Skip4	Dec 01	RMW330	51	Not tested
Skip1	Aug 02	30831	0	Not tested
Skip2	Aug 02	30832	0	Not tested
Skip3	Aug 02	30833	0	Not tested
Skip4	Aug 02	30834	86	Not tested
Skip1	Aug 03	971	3.28	Not tested
Skip2	Aug 03	972	1.56	Not tested
Skip3	Aug 03	973	0	Not tested
Skip4	Aug 03	974	9.42	Not tested

Summary based on data above:

Date	Feb-00							Feb-01	Feb-01	Nov-01	Nov-01	Aug-02	Aug-03
D.a.T.	1	2	4	8	10	**16**	**20**	48	**52**	**74**	**78**	**120**	**175**
Soil Cell 2a	306	1167	205	205		225	239		138		120		
Soil Cell 1	1366	520	155	435		766	150		78		10	0	3.28
Soil Cell 2	1097	563	268	192		0	102		190		0	0	1.56
Soil Cell 4	723	506	364		670			70		51		0	0
Soil Cell 3	364	141	126		210			25		35		86	9.42
Average	771	579	224	278	440	330	164	48	135	43	43	21	4

D.a.T. = Days after treatment

Nov-01: emptied soil cells into skip